IDENTIFICATION AND CHARACTERIZATION OF COMPOUNDS
WITH ANTIVIRAL ACTIVITY AGAINST INFLUENZA VIRUSES

A dissertation submitted
to Kent State University in collaboration with the
Lerner Research Institute, Cleveland Clinic
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

by
Ana Carolina Vazquez

December, 2008
# TABLE OF CONTENTS

List of Figures ............................................................................................................................................ x

List of Tables ............................................................................................................................................ xiv

Acknowledgments ....................................................................................................................................... xv

Chapter I  Introduction .................................................................................................................................. 1

- Influenza .................................................................................................................................................. 1
- Influenza virus .......................................................................................................................................... 2
  - Pathogenesis .......................................................................................................................................... 3
- Structure .................................................................................................................................................. 4
  - Hemagglutinin ....................................................................................................................................... 7
  - Neuraminidase ....................................................................................................................................... 8
  - M2 Protein ........................................................................................................................................... 9
- Reproductive cycle ................................................................................................................................. 9
  - Virus entry into host cell ....................................................................................................................... 10
  - Virus transcription ............................................................................................................................ 12
  - Viral genome replication ..................................................................................................................... 13
  - Virus assembly and release ................................................................................................................ 13
- Viral lethality of influenza viruses ........................................................................................................ 14
- Pandemic influenza .............................................................................................................................. 15
1.3.2 Conformational change of HA ............................................................55

1.4 Viral uncoating ..........................................................60

1.5 Inhibitors of viral RNA synthesis .................................62

1.5.1 RNA transcription ..............................................62

1.5.2 Synthesis of nucleic acids .................................68

1.6 Inhibitors of viral progeny release ...........................72

1.7 Additional agents with potential anti-influenza therapeutic effect ........................................75

1.7.1 Immune system stimulation .....................................75

1.7.2 RNA interfering .................................................76

Chapter II  Screening of small molecule library for the identification of potential anti-influenza virus compounds ........................................78

Introduction ........................................................................................78

Materials and Methods .......................................................................80

Results ................................................................................................85

Discussion ........................................................................................102

Chapter III  Characterization of anti-influenza properties of compounds with antiviral activity against influenza virus .........................................111

Introduction ........................................................................................111
Materials and Methods.................................................................112
Results............................................................................................128
Discussion........................................................................................174

Chapter IV  Summary and future directions.................................185
Summary........................................................................................185
Future directions ............................................................................187

References........................................................................................201
Appendix. Abbreviations.................................................................229
LIST OF FIGURES

Chapter I

Fig 1.1 Structure of influenza A virus ................................................................. 5
Fig 1.2 Influenza virus reproductive cycle ............................................................ 11
Fig 1.3 Sequence of antigenic shifts of the twentieth century ......................... 16
Fig 1.4 Process of drug discovery and development ............................................. 21
Fig 1.5 Chemical structures of currently approved M2 inhibitors ....................... 30
Fig 1.6 Mechanism of action of adamantane derivatives ...................................... 31
Fig 1.7 Chemical structures of currently approved NA inhibitors ....................... 40
Fig 1.8 Inhibition of influenza virus NA ................................................................. 41
Fig 1.9 Chemical structures of inhibitors active against the conformational change of influenza virus HA ................................................................. 57
Fig 1.10 Chemical structures of novel compounds targeting viral uncoating ....... 61
Fig 1.11 Chemical structures of inhibitors of influenza viral RNA transcription ...... 64
Fig 1.12 Representation of antisense oligonucleotide targeted at the influenza virus PB2 gene ................................................................. 69
Fig 1.13 Chemical structures of compounds that target the activity of IMPDH ...... 70
Fig 1.14 Chemical structures of novel NA inhibitors currently under study .......... 73
Chapter II

Fig 2.1  High throughput screening (HTS) system used to identify potential anti-influenza compounds .................................................................82

Fig 2.2  Z’ Value Determination ........................................................................88

Fig 2.3  Determination of drug susceptibility of 24 selected hits by cell protection assay .........................................................................................92

Fig 2.4  Structural Analogs of QMV-13 and QMV-15 ........................................98

Fig 2.5  Potential anti-influenza virus activity of structural analogs of QMV-13 and QMV-15 .................................................................................101

Fig 2.6  Summary of HTS of 34,000 small molecules library for anti-influenza compounds .........................................................................................106

Fig 2.7  Comparison among antiviral properties of the parental molecules (A) QMV-13 and (B) QMV-15 with their analogs .................................109

Chapter III

Fig 3.1  Drug susceptibility assays by different methods .......................................130

Fig 3.2  Viral plaque reduction assay in MDCK cells under a low viscosity overlay ...........................................................................................................132

Fig 3.3  CC\textsubscript{50} values of selected compounds in different immortalized human epithelial cell lines mimicking the respiratory tract ..............136

Fig 3.4  In vitro toxicology of lead compounds .......................................................140
Fig 3.5  Wild-type A/Texas/36/91 vs. oseltamivir-resistant A/Texas/36/91  ..........143

Fig 3.6  Antiviral activity against different RNA viruses ...........................................148

Fig. 3.7  Evaluation of antiviral activity against Human Immunodeficiency Virus

Type 1 (HIV-1) ........................................................................................................149

Fig. 3.8  Design of Time-of-Drug-Addition experiments ...........................................151

Fig. 3.9  Delay of treatment initiation on antiviral activity .......................................152

Fig. 3.10 M1 and M2 viral protein expression after different times of treatment

initiation .................................................................................................................154

Fig. 3.11 Design of Viral Protein Expression Experiments ........................................157

Fig. 3.12 Viral protein expression at different steps of the virus life cycle ..................158

Fig. 3.13 Virus yield reduction after high MOI infection in the presence of the

lead compounds ....................................................................................................161

Fig. 3.14 Design of Virus Yield Reduction assays after low MOI infection ..........163

Fig. 3.15 Virus yield reduction following multiple rounds of replication in the

presence of the lead compounds .........................................................................165

Fig. 3.16 Effect of lead compounds on the NA activity of influenza virus .............167

Fig. 3.17 Selection of drug-resistant influenza virus .............................................170

Fig. 3.18 Drug susceptibility determinations of serially passaged influenza viruses ..172

Fig. 3.19 Inhibition of the influenza virus reproductive cycle by antiviral drugs .....184
Chapter IV

Fig 4.1  Isobolograms for drug combination treatments of MDCK cell monolayers .................................................................195

Fig 4.2  Z’ Value Determination of ELVIRA®Flu HTS system ........................................200
LIST OF TABLES

Chapter II
Table 2.1 IC_{50}, CC_{50} and TI of 12 pre-selected hits.................................................................95

Chapter III
Table 3.1 Influenza A and B laboratory-adapted, clinical isolates and drug-resistant viruses used in this study...............................................................115
Table 3.2 IC_{50} Values of lead compounds and control drugs by different methods.................................................................131
Table 3.3 CC_{50} determinations of selected compounds in cell lines used for their characterization.................................................................135
Table 3.4 IC_{50} values of lead compounds against different strains of influenza A virus .................................................................142
Table 3.5 IC_{50} values of lead compounds against different strains of influenza B virus.................................................................145
Table 3.6 Antiretroviral activity of compounds QMV-15 and QMV-15A.............150

Chapter IV
Table 4.1 Effect of drug combinations on MDCK cells survival after infection with influenza virus A/WSN/33.................................................................192
ACKNOWLEDGMENTS

The journey in the pursuit of my Ph.D. degree was far more than educational; it enriched my life and broadened my perspectives. Along this experience I was lucky enough to be guided by Dr. Miguel E. Quiñones-Mateu, whose advice always came with a friendly smile but also loaded with high expectations that helped me get to where I am today. I thank him for sharing all his knowledge with me and for giving me the opportunity to learn from expert researchers from our field.

Ever since I started in my lab it was Dr. Jan Weber who very patiently answered all my questions, introduced me to new techniques and listened to my complaints when things did not turn out as expected. I am grateful to have shared with him and Jitka all these years in the lab. I would also like to thank my former lab-mates at the Cleveland Clinic Muneer Mirza, Oyebisi Jegede, and Michael Marotta who also were always there to help me during my learning process.

Achieving this goal was possible thanks to the School of Biomedical Sciences of Kent State University. I also extend my gratitude to the members of my dissertation committee Dr. Robert T. Heath, Dr. Philip E. Pellett, Dr. Oscar Rocha, Dr. Kenneth S. Rosenthal, and Dr. Christopher J. Woolverton for offering their guidance and advice when I needed it.
Essential contribution was provided by Diagnostic Hybrids Inc. at different stages of my research work, for which I particularly thank Drs. David Scholl, Heather Tomlinson, Yunsheng Li, and Jennifer Pappas. Also, I would like to thank Dr. Andrei Gudkov, Dr. Mikhail Chernov, and Anatoliy Prokvolit for facilitating the access to the library of small molecules; Drs. Christopher Roberts, Larisa Gubareva, and Ruben Donis for sharing important reagents and techniques; and Dr. Andrew Pekosz and Jason A. Ader for the invaluable training and continuous feedback on influenza virus.

Nothing that I could write in this page could possibly reflect my endless gratitude to my family. Mamá and Papá, thank you for giving me all that I needed and more, not only physically but morally and spiritually. Ana Maria and Alberto, this manuscript would not have been the same without your unconditional help and admirable creativity. Dragos, your infinite patience and kindness meant everything along these years. Thank you all for believing in me and being there every time I needed you, even when physically far away.
INTRODUCTION

Influenza

Influenza is a highly contagious, acute respiratory disease that has afflicted humans since ancient times. With influenza viruses as causative agents, this infectious viral illness is a major cause of morbidity and mortality in communities (epidemics) and worldwide (pandemics). Its rapid propagation is normally due to high infectivity of the virus, short incubation period as well as high titer in respiratory secretions during the shedding phase. The primary way of spread of human influenza is by virus-laden respiratory secretions during coughing and sneezing, with direct or indirect contact representing other possible modes of transmission. After virus replication and spread of infection throughout the upper and lower airways, virus is shed in nasopharyngeal secretions for 5 to 10 days. Following an incubation period of 1 to 4 days, a broad spectrum of symptoms also known as “flulike” symptoms, comprise febrile illness accompanied by variable respiratory disease with or without systemic features, including cough, rhinorrhea, headache, malaise and myalgia. Multi-system complications affecting lungs, heart, brain, liver kidney and muscle, as well as death are also possible outcomes due to either primary viral infection or secondary bacterial pneumonia (143, 143, 211, 225, 263).
During a typical year approximately 20% of children and 5% of adults have symptomatic influenza infection worldwide (264). In the United States, complications from influenza such as pneumonia and cardiopulmonary or other chronic diseases lead to more than 200,000 hospitalizations (253) and about 30,000 to 50,000 deaths per year (36, 246). The risk for the complications mentioned is higher among elderly people (>65 years old), young children, and those with certain underlying medical conditions. In fact, about 63% of all hospitalizations occur among persons aged >65 years old from which 5-10% lead to fatal outcome (119). Hospitalization rates among children aged <24 months are comparable to rates reported among elderly (50, 129).

**Influenza Virus**

Member of the *Orthomixoviridae* family, influenza virus is an enveloped virus with a segmented negative-stranded RNA (ssRNA). Three different types of influenza (A, B, and C) can be distinguished by antigenic differences between their nucleocapsid (NP) and matrix (M) proteins. Also, influenza virus type A is further subtyped according to variations in the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (144).

According to the current nomenclature system, strains of influenza virus are designated with the type (based on the antigenic specificity of the NP antigen, i.e. A, B or C), host of origin (indicated only for non-human hosts), geographic location of first isolation, strain number, and year of isolation. For influenza A viruses the antigenic description (HA and NA) is provided in parenthesis following the strain designation (e.g.
A/PR/8/34 (H1N1), A/HongKong/8/68 (H3N2), A/Swine/Taiwan/70 (H3N2),
B/Russia/69, C/California/78) (286).

All three influenza viruses infect humans and cause disease; however, they have
different epidemiologic characteristics: (i) Type A viruses are the cause of severe
recurrent epidemic diseases with high mortality in humans; they can also infect other
mammals such as pigs, horses, seals, as well as a variety of domestic and wild birds (e.g.
swine, chickens, turkey, ducks, geese). Influenza A viruses are perpetuated in nature by
wild birds, especially shorebirds and waterfowl (52, 120, 159). Fifteen HA and nine NA
subtypes have been identified to date but only three types of HA (H1, H2 and H3) take
part in influenza A virus attachment to human cells, and two types of NA (N1 and N2)
are in charge of virus penetration (5, 212). (ii) Influenza B viruses display a higher
immunologic stability than those from type A. It has been isolated from seals, but most
commonly affects humans causing clusters of infection in closed populations (36, 197).
(iii) Influenza C virus is capable of infecting humans, dogs, and swine. Cases of human
illness are usually subclinical and therefore rarely reported (38, 226, 246).

Pathogenesis

Influenza virus infects epithelial cells of the upper respiratory tract, with the
possibility of spread to the lower tract to cause an even more significant pathology (116).
Rapid replication of the virus efficiently halts cell protein synthesis and induces apoptosis
(164), compromising the host’s natural protective mechanisms (i.e. mucus secretion,
ciliary action, and protease inhibition) and accumulating in the lumen of the respiratory
tract for easy spread to other hosts (22) (159, 186). Once the epithelial cells are infected, it is mainly the adaptive immune response that is responsible for virus clearance. Pro-inflammatory cytokines such as interleukin-6 (IL-6) and IFN-α are released early after infection, phenomenon that is directly associated with the appearance of clinical symptoms. This is followed by an increase of influenza-specific plasma cells, including effector CD8$^+$ T cells (cytolytic T lymphocytes, CTLs) and CD4$^+$ T cells, which not only have cytolytic activity but also contribute for the production of antibody by B cells, among other roles (137). Humoral antibodies to the HA, NA, NP, and M proteins are produced during infection with influenza A, but it is the antibody response specific to HA and NA glycoproteins that has a protective effect against the virus. This protection seems to have a limited role in the resolution of established infection, although it has been shown that anti-HA and -NA antibodies help in the restriction of virus replication (184). Certainly, this antibody response provides the host with protection against reinfection with the same strain of influenza virus.

**Structure**

Influenza virions exhibit a pleomorphic appearance with an average diameter of 120nm. In influenza A and B virions, nucleocapsid proteins (NP) encapsidate eight viral RNA (vRNA) segments of different sizes, each of which is associated with a polymerase complex comprised by polymerase B2 (PB2), polymerase B1 (PB1), and polymerase A (PA) proteins (135, 231) (Fig 1.1).
Figure 1.1 Structure of influenza A virus. Indicated in this diagram is the matrix protein (M1) surrounding the ribonucleoprotein complex (vRNP). This complex consists of the viral polymerases (PB1, PB2, and PA) associated to the nucleocapsid proteins (NP), which encapsidate the eight segments of viral genome. Also indicated in the figure are the NS2 protein (present in small amounts in the virions) and the integral membrane proteins hemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2).
In this heterotrimer or viral polymerase complex, PB1 has the polymerase and endonuclease activities (161, 265), PB2 binds to the 5’ methylated cap of host cell mRNA (227, 265), and PA is essential for vRNA synthesis (188) as well as for proteolytic activity (97).

The association of these four proteins (NP, PB1, PB2, and PA) as a whole represents the transcriptionally active form of the genome and it is known as the viral ribonucleoprotein complex (vRNP) (126). It is surrounded by the matrix protein (M1) which forms a layer underneath the lipid cell-derived envelope, providing rigidity to it and playing a key role in virus progeny assembly and budding (26). The M1 protein also takes part in export of the vRNP complex from the nucleus in combination with the NS2 or nuclear export protein (NEP), which is also present within the virion (195).

The eight gene segments of influenza A and B virus genome encode for ten proteins, nine of which are incorporated in the progeny virions. Six segments of the genome encode for one viral protein, the latter being PB2, PB1, PA, the surface glycoprotein hemagglutinin (HA), NP, and neuraminidase (NA) in that particular order from segments 1 to 6. Two open reading frames (ORFs) in segments 7 and 8 encode for M1 and M2 proteins and NS1 and NS2 proteins respectively (144, 231).

The structure of influenza C clearly diverges from that of influenza viruses A and B, having only seven genome segments and expressing a single surface glycoprotein (HEF; hemagglutinin, esterase, and fusion) that functionally replaces the HA and NA of influenza A and B (135, 217).
Because of the relevance of their functions during the virus life cycle and as preamble for the information discussed in this study, the viral proteins hemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2) are described more in detail in the following paragraphs.

**Hemagglutinin (HA)**

Hemagglutinin is the surface glycoprotein of influenza A and B virus with receptor-binding and membrane fusion activities. In addition, HA is the primary target for neutralizing antibodies, and changes in its antigenic structure are associated with influenza A virus epidemics (237). This glycoprotein is synthesized in the rough endoplasmic reticulum, and is transported to the cell surface via the Golgi apparatus. It is synthesized as a single precursor molecule (HA\textsubscript{0}) that needs to be cleaved into two subunits (HA\textsubscript{1} and HA\textsubscript{2}) in order for the virus to become infectious (53).

HA of human influenza viruses binds to moieties containing 5-N-acetylneuraminic acid (sialic acid) on the surface of host cells. Moreover, this glycoprotein will bind preferentially to \(\alpha\)-(2,6) or \(\alpha\)-(2,3)-linked sialic acids depending on the strain of the virus. The expression of these linkages differ among virus replication sites in humans (\(\alpha\)2,6), birds (\(\alpha\)2,3), pigs (\(\alpha\)2,6 and \(\alpha\)2,3) and others, thus explaining influenza’s species tropism (280).

When in its precursor form (HA\textsubscript{0}), the HA glycoprotein is a homotrimer with the hydrophobic fusion peptide hidden at the base of the molecule. It is the low pH of the endosomes that triggers the irreversible conformational change that results in two
disulfide-linked chains (HA₁ and HA₂) with a “coiled coil” of alpha helices that re-orientates the fusion peptide towards the target membrane (protruding from the HA molecule), followed by HA trimerization and grouping of HA trimers to form a fusion pore (32, 56).

**Neuraminidase (NA)**

Neuraminidase or NA, is a glycoprotein present on the surface of influenza A and B viruses. Its structure has been described as a homotetramer that forms a spike with a head domain protruding from the cell surface, a trans-membrane domain, and a small cytoplasmic tail (135). Besides constituting a primary target against which neutralizing antibodies are produced, functional viral neuraminidase (NA) acts as a receptor-destroying enzyme (sialidase) cleaving sialic acid from the HA molecules, other NA and oligosaccharides at the cell surface. For this, NA catalyzes the hydrolysis of specific glycosidic linkages (α₂,6 or α₂,3) between sialic acid and its adjacent carbohydrate moiety (86, 212).

In addition, NA has been shown to play a role in initial viral infection with the removal of sialyl residues on mucins and cilia (decoy receptors for HA). By evading these natural defenses of the respiratory tract, NA grants virions the access to functional receptors on surface membrane of target cells (142, 169).
**M2 Protein**

The influenza A virus M2 (matrix 2) protein is a homo-tetrameric protein that is associated with the membrane and is present in virions in low molar amounts (49). This integral membrane protein acts as a proton selective ion channel after virion internalization. The low pH of the late endosomes activates the trans-membrane (TM) domain of M2, opening the ion channel to allow inward proton currents that modulate the pH inside the virions (203). The latter phenomenon triggers the dissociation of the viral matrix protein M1 from the vRNP, as well as the low-pH-induced structural rearrangement of the mature forms of HA; thus triggering fusion and uncoating events.

The envelopes of influenza B and C viruses also contain ion channels, the NB and CM2 glycoproteins, respectively. However, they differ considerably from the influenza A M2 protein in their aminoacid sequences (280).

**Reproductive cycle**

From the moment when influenza virus encounters the epithelial cells of the respiratory mucosa, an exponential growth of virus titer takes place, reaching its peak after 2 to 3 days (7). Various studies of the kinetics of influenza virus infection have confirmed this exponential increase in the number of progeny virions during the first days of infection (7, 12, 24, 231). According to different structured models, it takes about 6h for a round of virus replication to take place, that is, from the moment when a cell gets infected until progeny virus is released (Fig 1.2) (7, 231). The latter is done from the apical surface of the cell, thus allowing spread from cell to cell along the respiratory tract.
in a localized manner, without the systemic spread of influenza virus in the host (280). Virus particles are also recognized by cells of the immune system, such as alveolar macrophages and circulating dendritic cells, which coincides with the decrease in virus titers until it is undetectable after 6 to 8 days post infection (7) (280).

**Virus entry into the host cell**

After its attachment to specific cell-surface receptors, influenza virus particles enter the target cell by the clathrin-dependent receptor-mediated endocytic pathway (231). Once the endosomal pH is decreased to the required levels (~5.0), the viral M2 ion channels are activated and the interior of the virions become acidic. After this, conformational changes in HA expose the fusion peptide, which brings the viral and endosomal membranes to closer proximity resulting in their fusion (75, 144, 249). Under the same low-pH conditions, the interactions between viral M1 protein and vRNPs are disrupted and the latter are released into the cytoplasm. Nuclear localization signals (NLS) present on NP proteins allows for the import of vRNPs into the nucleus for virus replication (194).
Figure 1.2 Influenza virus reproductive cycle. Infectious virions bind to sialic acids on the cell surface and are internalized by endocytosis. The low pH of the endosomes activates the viral M2 ion channels triggering fusion of the two membranes and release of the vRNA into the cytoplasm. After their import into the nucleus, virus transcription produces vmRNAs that are translated into viral proteins, some of which return to the nucleus where they take part in viral replication and regulation of gene expression. Newly synthesized vRNA are assembled into vRNPs and directed toward the cell membrane for the final virus assembly and release.
**Virus transcription**

Synthesis of vmRNA starts with the priming of influenza viral RNA transcription, a very distinctive process called “cap-snatching” where the viral endonuclease cleaves capped and methylated 5’ termini of host mRNA molecules. For this, NS1 binds capped cellular mRNA in the nucleus and the 5’-terminal 10-13 nucleotides are removed by NS2 (204). Elongation of influenza virus mRNA (vmRNA) is initiated by the polymerase complex (PB1, PB2, and PA) with the incorporation of nucleotide residues at the 3’-terminal hydroxyl groups of the resulting primers. Transcription continues to near the end of the vRNA template where the RNA-dependent RNA polymerase complex encounters a stretch of (U)-residues that induces the synthesis of a poly(A) tail and termination (28). Synthesis of these polyadenylated vmRNAs is controlled during infection according to the levels of production of each virus-specific polypeptide (235). Newly synthesized vmRNAs are transported from the nucleus to the cytoplasm via nuclear pores, followed by viral protein production by ribosomes. The translation machinery synthesizes new PB1, PB2, PA, NP, NS1, NS2, and M1 proteins that are transported to the nucleus to take part in the processes of transcription and viral genome replication, as well as M and NS vmRNAs splicing (149). Also, viral envelope proteins (M2, HA, and NA) are synthesized by ribosomes on the surface of the endoplasmic reticulum (ER) followed by their glycosilation in the host’s cell secretory pathway, after which they are transported and incorporated into the cell membrane (231).
**Viral genome replication**

During influenza virus replication a different type of complementary RNA (cRNA) is produced that includes the sequences complementary to the 5' end of the genome segments and are neither polyadenylated nor capped (235). These full-length positive strand anti-genome transcripts are produced in similar amounts during infection by means of the NP protein, which allows the polymerase complex to read through the polyadenylation and termination signals (49). The cRNA molecules synthesized serve as a template for the production of negative-sense genomic RNA (vRNA) (101, 102). The PA protein allows the latter process to be initiated without a primer, and the addition of NP is also required for the elongation of full-length vRNA segments (149).

**Virus assembly and release**

Influenza virus ribonucleoprotein (vRNP) complexes are assembled in the nucleus, where their coupling to M1 proteins also takes place. The latter event in conjunction with the NS2 (NEP) protein, and nuclear export signals (NES) on the NP proteins, induces export of vRNPs to the cytoplasm (207). Newly formed vRNP-M1 complexes are directed toward the cell membrane where M1 connects the vRNP and the cytoplasmic tails of M2, HA, and NA glycoproteins leading to the release of progeny virions with the help of the sialidase activity of NA (86, 212).
Variability of Influenza Viruses

The severity of the influenza disease will depend on prior immunologic experience with other antigenically related variants of the virus (5). It is approximately every 2 to 3 years that influenza viruses undergo minor antigenic changes in the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. These changes, called “antigenic drifts”, are generated by immune selection as the result of the accumulation of point mutations (substitutions, deletions, insertions) in the HA and NA genes. Even though the mutation frequency for these two genes is relatively low (~1/10^4 bases per replication cycle), the rapid antigenic drift is possible due to the lack of proofreading activity of the viral RNA polymerase, which is also relatively error prone (52, 159, 186). Antigenic drifts occur in all three types of influenza virus (A, B, C) resulting in the development of new strains of the virus which are partially resistant to the antecedent strains due to the subtype and strain specific nature of the immunity to influenza virus A. Therefore, this type of antigenic change may result in an epidemic (38, 246).

A different antigenic phenomenon known as “antigenic shift” renders a totally new virus strain (new HA and possibly NA) that may result in a worldwide pandemic if the virus can be transmitted from person to person. It occurs only among influenza A viruses in at least two possible ways: (i) when an exchange of gene segments or genetic reassortment takes place after a host cell is co-infected with two strains of influenza A
virus, each from a different host species, or (ii) when a strain is transmitted without reassortment from an animal reservoir to humans (5, 52, 75, 186).

**Pandemic influenza**

**Pandemics of the twentieth century**

Although infrequent, antigenic shifts can generate outsized proportions of severe and fatal cases. That was the case of the H1N1 1918 pandemic that resulted in 20-40 million deaths worldwide, as well as the subsequent H2N2 1957 Asian (replacement of H1N1 subtype), and the H3N2 1968 Hong Kong (replacement of H2N2) pandemics, also associated with high levels of global mortality (52, 105) (Fig 1.3). The origin of the H1N1 1918 pandemic strain has been associated with avian sources, same as the H2N2 1957 and H3N2 1968 pandemics which have been shown to be reassortant viruses with HA, NA and PB1 genes from avian influenza A viruses (52, 159).

On the other hand, the described antigenic shift mechanism has been shortcut by some avian strains of influenza A capable of causing an outbreak of disease in humans without the involvement of natural reassortment events. Outbreaks of this kind (poultry-to-human spread) have occurred in Hong Kong by avian influenza H5N1 in 1997 and H9N2 in 1999, with increasing recurrence and spread since 2003 (Fig 1.3). (52, 159, 198, 288).
Figure 1.3 Sequence of antigenic shifts of the twentieth century. This figure depicts the chronologic emergence of influenza pandemics since 1918 until 2006 when the recurrence of poultry-to-human infections with the highly pathogenic avian influenza A/H5N1 was still reported. Figure adapted from reference (37).
Potential impact of the next pandemic

The World Health Organization (WHO) acknowledged in 2005 the prolonged existence of an influenza virus of pandemic potential (282). Since 2003, an ongoing outbreak of H5N1 avian influenza virus has infected humans in Asia, Europe and Africa, often with fatal results. About 90% of the cases reported so far have a history of exposure to infected poultry, even though epidemiological evidence suggests that only minor alterations in the avian strains will be sufficient to generate a virus capable of the feared human-to-human transmission (159).

Based on extrapolation of past pandemic experiences the Centers for Disease Control and Prevention (CDC) has estimated that, in the United States alone, a novel influenza strain capable of sustained person-to-person transmission (pandemic) could affect from 15 to 35 percent of the population, killing 89,000 to 207,000 people and generating an economic impact of US$71 to $167 billion. Moreover, the U.S. Department of Health and Human Services (HHS) applied the death rate from 1918 pandemic to the current population and predicted an approximate of 180 to 360 million deaths worldwide and 1.9 million deaths in the United States (117, 118, 138, 175). The economic impact of such a global epidemic would certainly overwhelm the United States care system causing a severe service disruption among other crisis (105).

Because there is no palpable resource to completely control an influenza pandemic, the use of vaccines and antiviral drugs together with quarantine and masks will be indispensable in order to mitigate the socio-economic impact (198). Nevertheless, even if the production of a vaccine started the same day that the pandemic is declared it
would take from four to six months to produce the first doses (281). Hence, the appropriate use of antiviral agents would be the only feasible option at the early stages of an influenza pandemic (69).

Two classes of antivirals are currently licensed by the Food and Drug Administration (FDA) for use in the United States, the M2 proton channel inhibitors (amantadine and rimantadine) and the neuraminidase inhibitors (zanamivir and oseltamivir)(71). Both have proven to afford successful chemoprophylaxis and treatment of influenza infections (191). However, due to the obvious need for additional antiviral agents, future alternative treatments targeting different stages of the viral replicative cycle are under development.

**Current trends in drug discovery**

In order for a novel compound to become a marketed drug a long and costly process has to be overcome. Starting from the rational selection of potential targets (243), researchers have to elucidate the intricate details of the disease to characterize and select a molecule. After this, they must efficiently screen large numbers of compounds to identify those with certain activity against that target. Once a “lead” is chosen, scientists have to figure out its mechanism of action as well as improve its physical-chemical properties to properly fit the standards of an ideal drug. Lastly, the ultimate test has to be passed when clinical trials are performed to confirm the effectiveness and safety of the future drug.
Methods to screen for anti-influenza virus agents

The complex process of drug development is nowadays simplified by the combination of diverse methods such as rational drug design, combinatorial chemistry, virtual screening, and experimental or high throughput screening (HTS) (Fig 1.4).

One of the most specific approaches is the one of the *rational drug design*, in which analysis of the receptor molecule structure and chemistry provides essential information for the design and further synthesis of highly selective molecules. All these are generally achieved with the help of special computer programs that probe the target molecule to predict the structure of compounds docking into the active site with the highest binding affinity (270). This structure-based design has already contributed to the development of important treatments like the anti-influenza drug “zanamivir” (Relenza) from GlaxoSmithKline (270) and human immunodeficiency virus (HIV) protease inhibitor “Ritonavir” from Abbott Laboratories (224).
Figure 1.4 Process of drug discovery and development. This flow chart designed by Hogan et al. describes the progression of stages that lead to an Investigational New Drug (IND), from the identification of “hits” and the tools currently available for that purpose, to the characterization of the lead compounds. Figure adapted from reference (122).
An alternative method used in the design of novel compounds is *combinatorial chemistry*, which generates large numbers of potential drug candidates by synthesizing structurally related molecules (80, 122). The creation of these compounds is based on the varied combination of different organic building blocks by solid-phase split-and-recombine methodologies. Combinatorial chemistry is a powerful tool for the construction of chemical libraries for random screens as well as for the optimization of lead compounds (122).

When it comes to screening large libraries of available compounds a widely used technique is *virtual screening*, in which computational methods dock every organic molecule from a database into the selected receptor, discarding those with low or no affinity (230). There are several phases in the process of “docking screens”, including the preparation of the compound database (e.g. removal of those with unwanted reactive or toxic groups), creation of the 3D pharmacophore (if narrowing the search based on the chemical similarity to a known ligand), preparation of 3D structure of selected target, molecular docking, as well as scoring (post-docking analyses) (163). This in silico approach is now commonly used in pharmaceutical research because of its accessibility and broad spectrum at little cost. In fact, the combination of virtual screening with the empiric approach has proven to narrow the expenses of the latter alone, particularly in those cases when the selected assay requires expensive reagents and extensive handling. Moreover, virtual and experimental screenings have been suggested to provide complementary benefits. Published data have shown that each technique can yield highly
diverse hits (i.e. selected compounds) displaying very different structures and chemical properties (68, 163, 268).

Experimental screening of chemical libraries, also known as *high throughput screening (HTS)*, seeks the identification of “primary hits” or compounds with potential activity against the target, which will eventually be followed by an optimization process with the use of medicinal chemistry (268, 272). The scale at which HTS are performed has changed throughout the years, having varied from tens of thousands (1990s) to the currently common hundred thousands of compounds per screen (268). Depending on the size of the library among other variables, HTS techniques can vary from semi-automated assays to entirely automated systems. Yet, independently of the screening strategy, the reliability of HTS assays must be evaluated in order to assure high levels of precision and reproducibility. A common measure of assay performance is $Z'$, a statistic value that takes into account both the “signal-to-background” ratio (signal dynamic range) and “signal-to-noise” ratio (variation) (176, 290). In practice, $Z'$ value standards for HTS establish that an assay can be considered excellent when its $Z'$ scores fall between 0.5 and 1, with 1 being “perfect” (290).

Recent developments in the field of drug discovery have provided researchers with a diverse spectrum of methods that in combination facilitate the efficient identification of drug-like compounds, with the potential to become novel treatments against a variety of targets.
**HTS Methods for the Identification of Anti-Influenza Virus Agents**

Since its adaptation to animal viruses by Dulbecco and Vogt in 1953, the plaque assay has been considered the gold standard for the measurement of viral susceptibility to antiviral drugs (144). However, this infectivity-based method is laborious, time consuming and somewhat subjective (number of plaques determined by hand); properties which make it unsuitable for its application on a large scale. In the search for an accurate, reproducible, and highly sensitive HTS assay for the identification of anti-influenza virus agents, a variety of methods have been proposed.

One of the broader approaches is the use of cell-based systems that assay the inhibition of virus-induced cell death. Utilization of this type of system as a HTS technique allows for the identification of potential influenza inhibitors while avoiding undesirable cytotoxicity (192). Examples of this type of approach are:

(i) *Colorimetric MTT (tetrazolium) assay* (124, 274), whose principle relies on the reduction of the yellow substrate (tetrazolium ring) by mitochondrial enzymes to yield a water-insoluble blue formazan product when incubated with metabolically active mammalian cells (182). This tetrazolium-based colorimetric method is very easy to perform and when automated it can quantitatively assay cytotoxicity and cell survival in a large scale (182).

(ii) *Lactate dehydrogenase (LDH) detection technique* also allows the measurement of influenza virus-induced cell damage (275). Measurement
of LDH in the cell culture is a direct indicative of cell degeneration given that it is a metabolic enzyme normally present in the cell cytoplasm (269).

(iii) Dye uptake methods such as neutral red have also been proposed as colorimetric indicators of cell viability for the screening of anti-influenza compounds (240). Neutral red is taken by living cells via non-ionic diffusion and is accumulated in the lysosomal compartments, staining them (34). The remaining color is read by colorimetry, allowing the identification of those compounds capable of inhibiting the influenza virus-induced cytopathic effect.

(iv) Crystal violet staining has also been adapted to an automated system that efficiently evaluates and calculates the anti-influenza activity of compounds with results correlating with the trusted plaque assay (221). For this, a solution of crystal violet in methanol is used for conveniently fixing and staining the nuclei based on the stain’s ability to bind DNA, followed by cell lysis, solubilization of the absorbed dye, and spectrophotometric quantification (83, 153, 218).

(v) A luminescent cell viability assay “CellTiter-Glo” (Promega Corporation, Madison, WI) has been validated for HTS measuring influenza virus-induced cell death (192). This method uses ATP-bioluminescence for the determination of cell viability in culture. Given the linear relationship between the amount of ATP (proportional to the number of metabolically active cells) and the luminescent signal, the protocol relies on direct cell
lysis and the reaction of released ATP with the luciferin-luciferase (reagent) for the emission of light and its further measurement by means of a luminometer (54).

Another tool that could be put in practice in screening for anti-influenza compounds is the use of virus-inducible reporter genes (167). The proposed system consists on Influenza A virus-detecting cell lines stably transfected with influenza A virus responsive reporter gene constructs FluA-luciferase (ELVIRA®FluA) and FluA-GFP (Diagnostic HYBRIDS Inc., Athens, OH.). In both cases, the influenza A virus NP protein was substituted by the luciferase or GFP open reading frames in the negative sense, conserving the 5’ and 3’ untranslated regions, and cloned between the human RNA polymerase promoter and murine RNA polymerase terminator. This allows the viral polymerase to recognize and replicate the RNA present in FluA luc or FluA GFP-expressing cells (167). Clearly, either one of these reporter cell lines offer a straightforward way for the evaluation of influenza sensitivity to large number of compounds as soon as 24 hours after infection in a multi-well format.

An alternative system correlating with the standard plaque reduction assay for the measurement of influenza virus in cell culture is the in situ cellular ELISA (Enzyme-Linked Immunosorbent Assay) (187). Execution of this method with antibodies directed to a broad spectrum of influenza virus strains can be done in 96-well plates and adapted to an automated system, making it an objective and reliable option for antiviral compound screening (16, 187).
Also, the adaptation of branched DNA (bDNA) technology to HTS has been proposed for the identification of novel anti-influenza compounds due to its high reproducibility and ease of application (271). Branched DNA is a signal amplification technique developed for the quantification of a targeted DNA or RNA in cell lysates and clinical samples (185, 267). Detection of negative strand RNA of multiple influenza strains using this method has been documented after the design of oligonucleotides for the Nucleoprotein gene (271). A 96-well plate assay was developed, in which viral RNA hybridizes to complementary oligonucleotides on the bDNA capture plates as well as to branched oligonucleotides. The latter are further hybridized to enzyme-conjugated oligonucleotides to emit a quantifiable luminescent signal (271). The bDNA assay showed correlation with the standard cytopathic effect and plaque assay when applied to cell cultures infected in the presence of known influenza inhibitors (271).

A variety of other methods for influenza antiviral drugs are available, each of them with the potential of serving as a tool for HTS after a possible optimization for faster and standardized performance. Such is the case of assays like flow cytometry analysis (174, 245), neuraminidase activity (163, 208), RNA hybridization (210), transcriptional activity (154), cap binding (123), among others.
Anti-influenza virus agents

Targets of Anti-Influenza Therapy

The goal during any antiviral treatment is to lower viral titers and consequently prevent cell damage (212). Potentially effective chemotherapeutic agents can get to accomplish this by targeting one of the virus-specific processes within the virus replicative cycle in virus-infected cells. For the purpose of reviewing the antiviral drugs according to their mode of action, the life cycle of influenza virus can be roughly divided into seven of these steps or processes (Fig 1.2): (I) virion binding to sialic-acid containing cellular surface glycoproteins and glycolipids by means of HA, (II) virus entry via receptor-mediated endocytosis, (III) fusion and uncoating of the viral nucleocapsid after M2 matrix ion channel-mediated acidification of the vesicle, (IV) transcription and replication of the viral RNA genome in the cell nucleus, (V) synthesis of viral proteins, (VI) assembly of virions at the plasma membrane, followed by (VII) budding and release from the cell surface mediated by NA sialidase activity (75, 151, 186).

Currently approved Anti-Influenza Agents

1.1 M2 inhibitors

The presence of the M2 ion channel in the envelope of the influenza A virus allows the M1 and vRNPs inside to become exposed to the low pH of the endosome, exposure that is necessary for the fusion and uncoating events to take place (75, 144, 249, 280).
Two adamantane derivatives (amantadine and rimantadine) (Fig. 1.5) currently licensed for antiviral indications in the United States prevent virus replication in the infected cell by blocking the function of the influenza A virus M2 protein (273) (Fig. 1.6).

Surveillance reports gathered during the 2005-06 influenza season by the Center of Disease Control and Prevention (CDC) indicated high levels of resistance to amantadine and rimantadine. Based on these data and after thorough antiviral testing, the CDC issued a Health Alert in January 2006 with a recommendation against the use of the adamantanes for the treatment or prevention of influenza A infections in the United States for the remainder of the 2005-06 influenza season (35, 40). Recent reports from the CDC indicate that this recommendation remains effective (2007-08 influenza season) until susceptibility of influenza A viruses to these drugs is reestablished (73).
Figure 1.5 Chemical structures of currently approved M2 inhibitors A) Amantadine, and B) Rimantadine.
Figure 1.6 Mechanism of action of adamantane derivatives. The specific effect of M2 inhibitors amantadine and rimantadine is described in this figure by the inhibition of viral uncoating that result from the blockage of H⁺ influx through the virus ion channel.
1.1.1 Amantadine

Amantadine (l-adamantamine hydrochloride) is a primary amine with a characteristic tricyclic ten carbon ring (Fig. 1.6). Its hydrocarbon derivative origin gives it highly lipophilic properties which facilitate its absorption (258). The ability of this adamantan(amine) derivative to inhibit influenza A virus in tissue culture and animal models was described in the 1960s after its identification by traditional biologic screening assays (59, 220). After numerous clinical trials the use of amantadine against type A (H2N2) strains of influenza was licensed in 1966 in the United States. Later on, with the emergence of new influenza A subtypes and the potential threat of swine influenza, amantadine was finally approved by the FDA for prophylaxis and treatment of all type influenza A viruses (1). Currently, amantadine can be found in the market under the commercial name of “Symmetrel” from Endo Pharms, and as generic drug (amantadine hydrochloride) from USL Pharma, Sandoz, Actavis Mid Atlantic, Teva Pharms, Mikart, Hi Tech Pharma, Pharm Assoc, Morton Grove, Carolina Medcl, Silarx, Actavis Totowa, and Vintage (71).

General Pharmacology:

Amantadine is a lysosomotropic agent that acts by blocking the virus M2 protein ion channel, preventing protons from entering the virion and therefore inhibiting the uncoating of the influenza A virus (236, 273). It has also been documented that amantadine has an effect at later stages of the replicative cycle of the virus. Generation of amantadine-resistant influenza viruses containing mutations in the HA that allow it to mature at higher
pH has indicated that this drug has the ability to raise the endosomal pH when used at high concentrations (57). Additionally, various studies have indicated that treatment with amantadine can alter the ionic environment of the trans-Golgi network inducing a premature maturation of HA. The latter phenomenon renders the fusogenic form of HA to aggregate while in the exocytic pathway and hence prevent virus release from the transport vesicles (46, 273).

Amantadine is administered orally, displaying complete bioavailability and reaching the highest concentration in plasma in 2 to 6 hours. The drug exhibits an elimination half-life of 12 to 18 hours and is excreted unchanged in urine without previous metabolism (23, 69, 258). Even though amantadine has not been proven to cause serious renal, hepatic or hematopoietic toxicity, a series of adverse effects at the usual dosage have been documented (258). Side effects reported most frequently are of gastrointestinal and central nervous system (CNS) nature. The former include nausea, vomiting, diarrhea, constipation and loss of appetite; and the latter reflect CNS stimulation such as insomnia, nervousness, dizziness, and difficulty concentrating (17, 135, 258). One or more of these adverse effects occurred in 33% of young adults after four weeks of treatment with amantadine in a double-blind profilaxis study performed with eighty-eight subjects (31). This trial reported the appearance of symptoms during the first four days of treatment, as well as the reversibility of side effects once it was discontinued (31).
Treatment and Prophylactic Efficacy:

All known human influenza A subtypes (H1N1, H2N2, H3N2) have been shown to be susceptible to amantadine, as opposed to influenza B virus, which due to the lack of M2 protein cannot be targeted by this drug (160). Clinical studies have reported that treatment of influenza A virus infection with amantadine can shorten the disease by one day but no effect on nasal shedding could be confirmed. On the other hand, the protective efficacy of this drug has been evaluated in a number of placebo-controlled trials resulting in the prevention of 61% of influenza A cases (130, 131).

Although preseason immunization is the principal means for preventing influenza-related morbidity and mortality (36), prophylaxis with amantadine is an alternative if the vaccine is not available or early vaccination is not feasible. In cases like these, prophylaxis is indicated only for those at greatest risk for complications. However, when substantial antigenic differences from the epidemic strain are detected, amantadine is recommended for prophylaxis in all high-risk individuals whether or not they have received immunization. Initiation of prophylactic treatment is recommended once the evidence of influenza A virus in the region is confirmed and following exposure. Amantadine should be administered for the duration of the exposure risk in order to prevent loss of protection (183).

Antiviral Resistance:

Phenotypic amantadine resistance of influenza A virus was confirmed by in vitro studies only a few years after the identification of this drug as a potential antiviral treatment
(47). Point mutations in the sequence coding for the aminoacid position 26, 27, 30, 31, or 34 of the trans-membrane of the M2 ion-channel protein was described later as the molecular changes conferring resistance to adamantanes (S31N, L26F, V27A, A30T, G34E) (103). These resistant viruses display an uncompromised ability to replicate and have been shown to be genetically stable and transmissible from person to person causing typical influenza illness (106, 140). The proportion of adamantane-resistant influenza viruses reported to the World Health Organization Collaborating Center for Surveillance, Epidemiology, and Control of Influenza at CDC increased from 0.4% during 1994-1995 to 12.3% during 2003-2004 (29). In the United States, the rate of resistance increased from 1.9% in 2004 to 14.5% during the first 6 month of the 2004-2005 influenza season (40). Continuous surveillance by CDC during the 2005-2006 season reported that 193 (92%) of 209 influenza A (H3N2) viruses isolated from patients in 26 states were resistant (30, 35, 73). Additionally, two (25%) of eight influenza A (H1N1) viruses tested contained the known aminoacid change at position 31 of the M2 protein (30). Records from the 2006-2007 season, as well as current data from the 2007-2008 (38, 39) influenza season do not show any encouraging evidence of the reestablishment of susceptibility to adamantanes among circulating strains (99.8% of influenza A (H3N2) and 10.8% of influenza A (H1N1) viruses are resistant to adamantanes). Therefore, clinicians are still being instructed to avoid the use of these antiviral drugs for the treatment or prophylaxis of influenza.

Recent studies propose that the increasing rate of resistance to adamantanes could be unrelated to drug selection pressure and instead be attributable to its interaction with advantageous mutations located elsewhere in the viral genome (233). Thus, it could be
argued that a complete interruption in the use of these drugs will lead to the re-emergence of susceptible strains endangering their usefulness during future influenza outbreaks.

1.1.2 Rimantadine

Rimantadine (alpha-methyl-1-adamantanemethylamine hydrochloride) is a closely related analogue of amantadine that shares the tricyclic structure but differs in the substitution on the ring (Fig 1.5). Rimantadine was identified soon after its analogue and reported to be more active than amantadine against influenza A viruses \textit{in vitro} (261) as well as against experimentally induced influenza A infection in laboratory animals (223). Extensive epidemiologic studies were performed starting from 1969 in the Soviet Union, where rimantadine became the treatment of choice for influenza A infection (291). After a few drawbacks, rimantadine was finally licensed for use in the United States in 1993. The FDA authorized this drug for prophylaxis and treatment of influenza A in adults and for prophylaxis in children over 1 year of age (71). Currently available drugs with rimantadine as active ingredient are “Flumadine” from Forest Laboratories, and generics from Corepharma, Impax Laboratories, and Actavis Totowa (71).

\textbf{General Pharmacology:}

Understanding of the genetic basis of resistance to adamantane derivatives led to the confirmation that the mechanism of action of rimantadine is the inhibition of the M2 protein, thus preventing viral replication by blocking influenza A virus entry into the cells (14, 273).
Despite their similar structures, rimantadine and amantadine exhibit very different pharmacokinetic profiles. Rimantadine plasma concentrations average one half of amantadine after 6 hours, achieving higher concentrations in respiratory secretions. Following oral administration, rimantadine is extensively metabolized with renal elimination as the main pathway of excretion after a half-life of 25 to 36 hours (69, 258).

Studies of toxicity of rimantadine in humans have shown that it is better tolerated than amantadine when administered at equivalent doses (130). Moreover, multiple clinical trials performed in the Soviet Union did not find statistically significant differences in the frequencies of side effects between the rimantadine and the placebo groups except for sporadic symptoms of dyspepsia such as nausea and diarrhea (<1%) when given in prophylactic and therapeutic doses. (291).

In an effort to explain the significant differences between the toxicity profiles of rimantadine and amantadine, a double-blind, placebo-controlled study performed in the United States assessed the frequency of adverse symptoms in healthy individuals with similar plasma concentrations. The results show that the two adamantane derivatives have similar potential for side effects at comparable plasma concentrations, and that the main difference toxicity-wise resides in their rather different pharmacokinetics (111).

**Treatment and Prophylactic Efficacy:**

Rimantadine is an effective therapeutic agent in influenza A virus infection of all known subtypes of human strains (H1N1, H2N2, H3N2), but it is not active against influenza B virus or the avian flu (H5N1) strains that are currently capable of infecting
humans (160). A comprehensive review of a number of clinical trials published between 1966 and 2005 concluded that rimantadine has comparable efficacy to amantadine in treating symptoms of influenza A in healthy adults (130). Compared to placebo, rimantadine shortened the duration of fever by over one day, but had a low effect in interrupting transmission of the virus (130). The efficacy of this drug for prophylactic treatment was also concluded to be efficacious (72%) with significant preventive effect in both vaccinated and non-vaccinated populations. The prophylactic effect of rimantadine was extensively studied in Russia where it was concluded that this drug is more effective in preventing illness than infection with influenza A virus (283). Rimantadine and amantadine also showed comparable prophylactic effect with the difference that rimantadine was associated with fewer side effects (67, 130).

**Antiviral Resistance:**

One of the first reports on the emergence of rimantadine-resistant influenza A virus isolates was a double-blind, placebo-controlled study conducted in families using the drug for both prophylaxis and treatment during two influenza seasons. This study resulted not only in the confirmation of a rather rapid selection of drug-resistant isolates (as early as 2 days after initiation of treatment), but also showed that resistant viruses can be transmitted from person to person (110). Another study comparing rimantadine with acetaminophen for the treatment of children infected with influenza A virus indicated that 27% of the patients shed rimantadine-resistant virus during therapy (95).
Because rimantadine and amantadine have the same mechanism of action complete cross resistance occurs with these drugs, hence, aminoacid changes in the M2 protein (S31N, L26F, V27A, A30T, G34E) will completely abolish susceptibility to both drugs during prophylaxis (74, 103).

1.2 Neuramidinase inhibitors

Due to the presence of influenza virus HA on the same cell membrane during release of newly formed virions, cleavage of sialic acid residues by means of influenza virus NA becomes necessary in order to prevent the formation of viral aggregates and to allow spreading of virions to other host cells. Currently, two FDA approved antiviral drugs (oseltamivir and zanamivir) (Fig. 1.7) act by inhibiting influenza virus NA blocking the release of progeny virions, thereby reducing viral infectivity (60) (Fig. 1.8).
Figure 1.7 Chemical structures of currently approved NA inhibitors A) Oseltamivir, and B) Zanamivir.
Figure 1.8 Inhibition of influenza virus NA. Release of progeny virions from infected cells is prevented by NA inhibitors like oseltamivir and zanamivir by competing with the enzyme’s actual substrate (sialic acid). This accumulation of virions on the surface of infected cells halts the spread of infection within the host.
1.2.1 Oseltamivir

Oseltamivir phosphate (GS 4104) is an ethyl ester prodrug that requires ester hydrolysis by hepatic esterases to be converted to oseltamivir carboxylate (GS 4071), its active form (135, 173) (Fig. 1.7). The development of oseltamivir resulted from the combination of rational drug design and available high resolution x-ray crystal structures of sialic acid and its analogues bound to influenza A and B NA (139, 158). After the identification of GS 4071 as a potent NA inhibitor, modifications of the molecule had to be done in order to increase its oral bioavailability. It was the addition of a lipophilic alkyl group that resulted in a highly orally bioavailable form of GS 4071 (GS 4104) (158). Oseltamivir (Tamiflu, Roche) was licensed by the FDA in October 1999 for the treatment and prophylaxis of influenza infection (71).

General Pharmacology:

Oseltamivir is a potent inhibitor of the neuraminidase (NA) enzyme of the influenza viruses A and B. The lipophilic side chain of the active metabolite interacts with a hydrophobic pocket in the region corresponding to the glycerol subsite of sialic acid, thus competing with the enzyme’s actual substrate (49, 139).

After oral administration, oseltamivir is well absorbed from the gastrointestinal tract and converted to oseltamivir carboxylate. The latter has an absolute bioavailability of 80%, reaching maximum plasma concentrations after 3 to 4 hours with a remarkable volume of distribution, which allows it to effectively reach the potential sites of influenza virus
replication (135, 212). The elimination of oseltamivir is entirely renal in the form of the active drug, which has a half-life of approximately 6.7-8.2 hours (69, 173).

Treatment with oseltamivir does not appear to be associated with major adverse reactions. Mild side effects such as headaches, nausea, and vomiting were reported to have a 5% higher rate than the corresponding rates in placebo recipients during pre-licensing clinical trials (69). Yet, the incidence of the gastrointestinal side effects can be reduced by administering the medication with food (173). On the other hand, post-marketing use of oseltamivir has led to the identification of other possible side effects, such as rash, swelling of face or tongue, toxic epidermal necrolysis, hepatitis, abnormal liver function tests, arrhythmias, seizures, confusion, and aggravation of diabetes. However, in many cases it has not been possible to get a reliable estimation of the frequency of these side effects, as well as to establish a cause relationship to oseltamivir exposure (135).

**Treatment and Prophylactic Efficacy:**

Oseltamivir has been documented to be effective against the commonly circulating influenza virus strains with neuraminidases N1 or N2, as well as against influenza viruses with N3-N9 tested in vitro (69). Furthermore, the effectiveness of oseltamivir against recombinant influenza viruses possessing the HA and NA genes from the 1918 pandemic virus was confirmed in both tissue culture and mice, suggesting that this antiviral drug would be a useful strategy in the management of a re-emergent 1918 or 1918-like influenza virus (263).
In vitro studies have demonstrated the efficacy of oseltamivir in the treatment of infections caused by H5N1 (A/Hong Kong/156/97) and H9N2 (A/Hong Kong/1074/99) avian influenza viruses in mice (157). Moreover, prompt treatment with a neuraminidase inhibitor pending the results of diagnostic laboratory testing was recommended during a WHO Meeting on Case Management and Research on Human Influenza A/H5 (13). Also reviewed at this meeting were the results from murine studies indicating that influenza A (H5N1) requires higher oseltamivir doses and more prolonged administration to gain antiviral effects. Clinical studies evaluating the effects of treatments with twice the regular dose of oseltamivir in adults with uncomplicated human influenza did not find significantly higher antiviral benefits. Therefore, the proper dose and length of the treatment of avian influenza in humans are still to be assessed (13).

Oseltamivir has been associated with an early return to normal activities and a reduction in illness severity and complications (74). A placebo-controlled clinical trial in adults with naturally acquired febrile influenza reported the reduction of the disease by up to 1.5 days and the severity of illness by 38% when treatment with oseltamivir was started 36 hours of the onset of symptoms (260). Another study performed in Canada, Europe and China demonstrated that the earlier the initiation of oral oseltamivir therapy, the higher its therapeutic effects, indicating as much as 41% (3.1 days) reduction of the duration of illness when administered within the first 12h after fever onset (4).

Oseltamivir is indicated for the prophylaxis of influenza infection in adults and children aged 1 year and older (214). The use of oseltamivir for long term prophylaxis against influenza was evaluated in two placebo-controlled, double-blind trials during the
winter of 1997-1998 (108). A group of 1559 adults received either oseltamivir or placebo daily for six weeks during a peak period of local influenza activity. The neuraminidase inhibitor proved to be safe and effective, showing an average of 74% protective efficacy with a reduction of the risk of influenza from 4.8% (placebo group) to 1.2% among those assigned to oseltamivir (108). Additionally, a systematic review comprising published studies that had evaluated treatment or prevention of influenza with oseltamivir and zanamivir with data available before December 2001, showed that these drugs used prophylactically provided a relative reduction of 70-90% in the odds of developing flu (51). However, the authors of a more recent systematic review suggested that the available neuraminidase inhibitors should not be used routinely in seasonal influenza control since the data analyzed showed that they are ineffective in the prevention of asymptomatic infection or influenza-like illness (131). According to this publication, oseltamivir should only be used in a pandemic situation together with associated public health measures (131). Furthermore, a different study evaluating the efficacy of oseltamivir in post-exposure prophylaxis indicated that if administered early following infection the inhibition of viral replication by oseltamivir will effectively prevent the development of clinical influenza (89%) while prevention of initial viral infection is harder to achieve (63%) with this antiviral drug (278). Another post-exposure prophylaxis study confirms the effectiveness of oseltamivir in the prevention of influenza transmission in households reporting a protective efficacy of 68% (109).
Antiviral Resistance:

*In vitro* studies consisting of serial passages of virus in cell culture in the presence of increasing concentrations of oseltamivir have resulted in the generation of influenza A virus isolates with decreased susceptibility to this drug (93, 141, 171). Genetic analyses of the resulting inhibitor-resistant variants have identified mutations within the viral hemagglutinin (HA) or the neuraminidase (NA) genes or both. *In vitro* selection of NA mutant viruses requires prolonged passage in cell culture and result in substitutions such as H274Y, and I222T in influenza A N1; I222T and R292K in influenza A N2; and E119V, R292K and R305Q in avian influenza A N9. Although the viral NA is the known target for oseltamivir, mutations in the HA gene such as A28T and R124M (A/ H3N2) were obtained first within fewer passages (135, 214). These mutations are located in residues close to those involved in receptor binding, thus, compromising the sialic acid-binding activity of HA and reducing the dependence for the sialidase activity of viral NA (93, 172).

Whether these oseltamivir-resistant variants can emerge and be transmitted in vivo following treatment is a matter of thorough investigation. One of the initial approaches in monitoring this issue was the establishment of the Neuraminidase Inhibitor Susceptibility Network (NISN) in 1999 (181). More than 1000 clinical influenza specimens isolated from the 3 years before introduction of the NAIs (1996 to 1999) were screened for susceptibilities to oseltamivir and zanamivir showing no evidence of naturally occurring resistance to these drugs (170). Following a similar pattern for the collection of clinical samples from around the world (WHO influenza surveillance network), susceptibility to the NAIs of 2287 isolates recovered from 1999 to 2002 was evaluated (181). This study,
performed in viruses from the first three years of NAIs use, revealed a decrease (>10-fold) in susceptibility to oseltamivir in eight viruses (0.33%), none of which were obtained from individuals known to have had treatment with NAIs (181).

During the following years, incidence of development of oseltamivir-resistant strains of influenza virus was reported to be as low as 0.4-1% among the adult population and 4-8% in children (72). However, an 18% frequency rate of resistance in children was reported in a clinical study performed in Japan using more sensitive detection techniques (141).

Treatment of naturally acquired infection with influenza virus has been followed by the emergence of the H274Y substitution in neuraminidase N1 and E119V and R292K in N2 (214). Viral strains containing the latter mutation were 10,000-fold less infectious than wild-type virus in a mouse model. This and other studies have suggested that the defects caused by mutations in catalytic sites of NA compromise viral fitness by reducing enzyme activity and stability (33, 135, 289). However, a recent study performed in Europe by the European Surveillance Network for Vigilance against Viral Resistance (VIRGIL) detected A/H1N1 oseltamivir-resistant variants bearing the H274Y mutation with the ability to transmit between individuals (2). Moreover, another study on variant isolates has reported that mutations in framework residues such as E119V confer resistance to oseltamivir without compromising the replicative capacity and transmissibility of the virus (72).

Surveillance by the CDC during the 2007-2008 influenza season in the United States reported that as of week 20 (May 17, 2008) 10.9% of influenza A viruses tested were oseltamivir-resistant. All the resistant strains were H1N1, while no H3N2 or B viruses were
identified as resistant to oseltamivir, and 100% of resistant variants tested were sensitive to zanamivir (39). Influenza surveillance in Europe during the same season (as of January 2008) detected A/H1N1 oseltamivir-resistant viruses in nine countries, indicating high frequency rates in Norway (70%), France (17%), Germany (7%), and the United Kingdom (5%), with all of them bearing the H274Y mutation in NA (2). Furthermore, resistance to oseltamivir has been documented during treatment of influenza A (H5N1) infection, the most recent report being two of eight Vietnamese patients with a H274Y substitution in the NA gene (61).

Emergence of oseltamivir-resistant influenza B viruses has also been described although less frequently than resistant influenza A viruses. A clinical study performed in Japan during the 2004-2005 influenza season revealed that 1.4% of the children treated with oseltamivir developed a G402S NA substitution, but more importantly, the same study identified 1.7% resistant influenza B variants from untreated patients, suggesting the likelihood of transmission within communities and families (100).

1.2.2 Zanamivir

Zanamivir (GG167) or 5-acetamido-4-guanidino-6-(1,2,3-trihydroxypropyl)-5,6-dihydro-4H-pyran-2-carboxylic acid (Fig.1.7) is an analogue of sialic acid which potent activity results from the substitution of a hydroxyl group (C-4 atom) by a guanidine group. This group confers zanamivir a tight affinity for the active site of the viral enzyme NA (69). The discovery of zanamivir in 1989 was the outcome of computer-assisted design based on the crystal structure of the influenza NA active site resulting in the first potent,
selective NA inhibitor (270). Zanamivir has been commercially known as “Relenza” (GlaxoSmithKline) since 1999 when it was approved by the FDA for marketing in the United States followed by its approval in other seventy countries (71).

**General Pharmacology:**

Zanamivir is a selective inhibitor of influenza NA. This drug competitively blocks NA preventing the sialic acid cleavage thus reducing the release of progeny virions from the cell surface and subsequent viral spread (72, 173).

The characteristic positively-charged guanidine group of zanamivir confers low bioavailability to the drug (2%, range 1%-5%), therefore it is administered in the form of a powder to the respiratory tract by oral inhalation using a specifically designed breath-activated device called “Diskhaler” (69, 212). This way 10-20% of the drug effectively reaches the lungs and 4% to 17% of the inhaled dose is systemically absorbed with peak serum concentrations being reached in 1 to 2 hours (135). Zanamivir is not metabolized and it follows renal excretion, which lowers the possibility of serious adverse effects.

Clinical trials in adults and adolescents documented considerably low incidence of adverse reactions (1%-3%) such as headaches, diarrhea, nausea, cough, sinusitis, dizziness, among others (84). Furthermore, voluntary reports of side effects during post-marketing use of zanamivir include allergic reactions (hypersensitivity to the drug), delirium, confusion, agitation, arrhythmias, seizures, among others. Yet, due to the nature of these toxicity reports a reliable estimation of their frequency or direct cause relationship to zanamivir are not possible (84, 135). Nevertheless, inhalation of zanamivir has been
associated with bronchospasm and a decline in lung function in some patients with underlying airways disease such as bronchial asthma and obstructive pulmonary disease (69).

**Treatment and Prophylactic Efficacy:**

Clinical influenza isolates of subtypes H1N1, H3N2 and influenza B proved to be susceptible to the inhibitory effect of zanamivir when tested *in vitro* (69). Also, the antiviral activity of this drug has been confirmed *in vitro* against recombinant influenza viruses displaying the NA and HA segments of the 1918 pandemic virus (263), as well as other pandemic strains such as H2N2 (1957), H3N2 (1968), and H1N1 (1977), and avian H5N1, H6N1 and H9N2 strains (157).

Without showing a significant difference between cases of influenza A compared with B zanamivir reduces the time of alleviation by 1-1.5 days, also shortening the duration of viral shedding when treatment is initiated 48 hours after the onset of clinical symptoms (84). In addition, results from a series of phase III trials reported a 22% reduction in complications compared to a 29% in the placebo group (69).

Zanamivir has been confirmed to be effective in the prevention of naturally occurring influenza illness in multiple prophylaxis studies including post-exposure prophylaxis in households (79-81% efficacy) and seasonal prophylaxis studies during community outbreaks (60-83%) (84, 173).
Antiviral Resistance

Resistance to zanamivir has been induced in influenza A and B in vitro following extensive virus passage in the presence of the drug. After isolation and sequencing of the resistant viruses, changes in both HA and NA protein sequences were identified. HA mutations (K68R, G75E, E114K, N145S, S165N, S186F, N199S, K222T) are generated within fewer passages and the viruses produced display less affinity for sialyl receptors, which makes them less dependent on their NA activity (212). On the other hand, mutations within the NA have been identified in residues E119 and R292 (E119G/A/D and R292K) (89). The importance of the latter residue for substrate binding and stabilization of the transition state supports the findings that viruses generated in vitro containing the R292K substitution have less NA activity and infectivity than wild type virus in mice and ferret (91, 128, 289). Moreover, these results have been confirmed with the generation by reverse genetics of H3N2 viruses containing the H274Y, R292K, E119V and E119D mutations, which showed a significantly reduced ability to replicate in vitro, suggesting very low chances for their human-to-human transmissibility (292).

Even thought the generation of zanamivir resistant variants in vitro has been readily documented, no resistant virus has yet been isolated from immunocompetent patients receiving zanamivir (292) (107). Even after large numbers of clinical trials with this drug, the only case of clinical resistance to date has been a severely immunocompromised child with influenza B virus who received a delayed and prolonged treatment with zanamivir. The virus isolated carried mutations in both HA and NA genes. However, extensive
phenotypic analysis of this variant \textit{in vitro} has reported that its replicative capacity was more sensitive to zanamivir than the virus at the beginning of treatment (74) (90).

Zanamivir retains full activity against most but not all oseltamivir-resistant variants (177). Cross-resistance between the two NA inhibitors has been observed in cell culture, although in some of those cases the zanamivir-resistant mutations occurred at the same amino acid positions as in the clinical isolates resistant to oseltamivir (84). However, it has been documented that mutations on framework residues implicated in the stabilization of the active site structure such as E119 do not induce cross-resistance between oseltamivir and zanamivir (72).

Furthermore, there is evidence for different patterns of susceptibility and cross-resistance between NA inhibitors. Zanamivir is more potent in inhibiting NA activity in N2, N3, N6, N7, and N9 subtypes than oseltamivir while N1, N4, N5, and N8 are more sensitive to oseltamivir than to zanamivir. Inconsistencies in the inhibitory effects of zanamivir and oseltamivir among the different NA subtypes are attributed to amino acid substitutions surrounding the NA enzyme active center, suggesting that those residues involved in NA resistance in one group could not necessarily be related to inhibitor resistance in another group (72, 87).

\textbf{Antiviral agents with therapeutic potential against influenza}

Intense research is currently underway for the development of antiviral drugs that would expand the availability of influenza prophylactic and therapeutic treatments. Such goal becomes of outmost importance when considering the ineffectiveness of the M2
inhibitors against 99.8% to 10.8% of the circulating influenza strains (H3N2 and H1N1, respectively) and the increasing emergence of oseltamivir-resistant variants described in the previous sections. In response to this situation, known molecular targets as well as recently discovered and unexplored ones, are the focus of attention and starting point of scientist around the world. The following overview covers only part of those compounds that have been shown to exert promising antiviral effects against influenza and are currently under study for the examination of their therapeutic potential.

1.3 Inhibitors of influenza virus binding and fusion

1.3.1 Binding of HA to sialic acid

Blocking the binding of influenza virion HA to sialic acid on the surface of target cells would efficiently prevent them from initial infection. Although development and approval of a drug with such a mechanism of action has not been accomplished yet, many attempts are on their way. One of them, cyanovirin-N (CV-N), a naturally occurring cyanobacterial lectin derived from Nostoc ellipsosporum, specifically binds to high-mannose oligosaccharides on the viral HA1 subunit neutralizing its infectivity (193). Studies in mice using a reassortant virus that was lethal to the animals yet sensitive to CV-N resulted in up to 100% survival and 1000-fold reduction in lung virus titer on day 3 of the infection (238). Similar results after studies in ferrets showed that CV-N could be useful for prophylaxis and early initiation of treatment of influenza virus infection (238). Another type of mannose-binding lectins inhibiting virus binding are the collectins,
which are known to be part of the several innate immune mechanisms that protect humans from early influenza infection (250). Collectins bind in a calcium-dependent manner both viral HA and NA, effectively inhibiting virus binding as well as the sialidase activity of NA, as characterized in detail following in vitro studies using wild-type and recombinant mutant collectin preparations (250). Suggested to directly interact with collectins in the host response to viruses are the peptides belonging to the human defensin family (251). Defensins, which are subdivided into three general classes: α- (155), β- (65), and θ-defensins (248), have been documented to inhibit influenza virus in vitro. The ability of these antimicrobial peptides to inhibit infection is attributed to their lectin-like properties, even though details on their mechanism of action remain evasive. According to this, previous studies have speculated that in the case of α-defensins and β-defensins it is their binding to virus receptors what exert their inhibitory activity (43, 55, 251). On the other hand, θ-defensins have been described to inhibit influenza virus infection by blocking membrane fusion mediated by the viral HA even after attaining its fusogenic conformation or inducing membrane fusion (156). However, the latter mechanism just like in the case of α- and β-defensins was suggested to be the result of a protective barricade of immobilized surface proteins due to their lectin-like properties (156).

Finally, a standardized elderberry extract, sambucol (SAM), was shown to reduce hemagglutination thus inhibiting replication of human influenza (248). After proving its antiviral potency against a wide variety of influenza A and B subtypes, including avian A/H5N1, SAM is undergoing clinical trials in Israel to evaluate safety and effectiveness in the resolution of human influenza illness (248).
1.3.2 Conformational change of HA

Conformational changes of HA at low-pH conditions are responsible for bringing the viral and endosomal membranes into proximity, allowing for their fusion to occur (249). Inhibition of this crucial step in the virus life cycle is a relevant goal in the development of novel anti-influenza drugs. Some of the potential fusion inhibitors currently under study are BMY-27709 (165), TBHQ (25), 180299 (244), CL385319 (205), PM523 (229). Compound **BMY-27709** (Fig 1.9) is a derivative of quinolizin-benzamide that, through the use of reassortants, drug-resistant, and transfectant viruses, has been shown to specifically inhibit H1 and H2 subtypes of influenza A virus by binding to the amino terminus of their HA2 and repressing their switch to the needed fusogenic state (166). Another compound reported to inhibit the conformational change in HA, and thus influenza virus infectivity, at low micromolar concentrations is *tert-butyl hydroquinone (TBHQ)* (Fig 1.9) (25). This fusion inhibitor is the most potent of the products of a structure-based design that resulted in a family of benzoquinones and hydroquinones. Thorough characterization of these compounds that included drug susceptibility determinations with strain X:31 influenza virus (subtype H3) and the development of resistant variants to each inhibitor showed that, like TBHQ, most of them inhibit the conformational change and membrane fusion activity of HA (121). Contrary to BMY-27709, subtype specificity of TBHQ has shown to be limited to H3 viruses, with very low activity against the H2 subtype and no effect on the fusion by influenza virus of the H1 subtype (121).
**7-Ketopodocarpate (180299)** (Fig 1.9) was described as a potent inhibitor of multicycle replication of influenza A/Kawasaki/86 (H1N1) *in vitro*. (244). Following selection of drug-resistant variants, reassortants between wild-type and resistant viruses were generated and drug susceptibility tests performed indicated that the 180299-resistant phenotype is only conferred by mutations in HA (244). Moreover, a dose-dependent inhibition of wild-type influenza A/Kawasaki/86-infected MDCK cells fusion to human erythrocytes confirmed the ability of 180299 to prevent low-pH-induced change of HA to its fusogenic conformation (244).

Identified by Plotch et.al. after the screening of a chemical library, **N-substituted piperidine (CL385319)** (Fig 1.9) has been shown to inhibit replication of H1 and H2 subtypes of influenza A (205). Virus protein expression was inhibited by CL385319 when added before or at the time of infection but not 30 minutes later, pointing at virus uncoating as the possible step of the cycle targeted. Confirmation of the latter was obtained from the inhibition of fusogenic activity of HA during cell-cell fusion assays. Also, CL385319-resistant viruses allowed for the identification of its molecular target and computer-assisted modeling to further characterize this fusion inhibitor (205).
Figure 1.9 Chemical structures of inhibitors active against the conformational change of influenza virus HA. A) BMY-27709, B) TBHQ, C) 180299, D) CL385319, and E) Futhan.
Interestingly, the mutations in the HA gene conferring resistance to BMY-27709, TBHQ, 180299, and CL385319 were similar in that they mapped to regions close to a common gap between the HA1 and HA2 molecules, indicating that these fusion inhibitors may be exerting their effect by sitting in that region and directly blocking the movement of the fusion peptide (166, 228).

A very different compound targeting the conformational changes of influenza HA is **PM-523**, a polyoxometalate which structure is comprised by clusters of inorganic molecules including oxide anions and early-transition metal cations (229). Inhibition of the virus envelope-cell membrane fusion of influenza A virus by PM-523 was determined by fluorescence dequenching tests performed using rhodamine-labelled virus particles and MDCK cells (228). Further characterization of the mechanism of action of this compound was possible with the selection of PM523-resistant viruses which allowed for the identification of its molecular target. Albeit its structural differences compared to the drug candidates previously described, PM-523 has also been shown to be interfering with the conformational change that gives raise to the fusogenic peptide (228). This antiviral activity has been already tested *in vivo* alone and in combination with ribavirin, an RNA-synthesis inhibitor, showing very promising results (228).

Another approach that has been explored for the inhibition of the influenza virus RNPs release into the cytoplasm of infected cells is the inhibition of the HA proteolytic cleavage. Although a wide variety of trypsin-like enzymes have been proposed, selective targeting of the influenza HA is necessary for a drug to be used in this kind of treatment. Someya et.al. have identified two candidates, **futhan** (6-amidino-2-naphthyl p-
guanidinobenzoate) (Fig 1.9) (241) and *anti-cathepsin B IgG antibody* (242), that display this property *in vitro*.

### 1.4 Viral uncoating

Acidification of influenza virus insides is mediated by the activation of viral M2 ion channels after a drop in the endosomal pH. It is this phenomenon that triggers the conformational changes in HA, as well as the disruption of interactions between viral M1 protein and vRNPs to result in uncoating of the virus and release of the latter into the cytoplasm. (75, 144, 249). Even though the ion activity of viral M2 is already targeted by the approved anti-influenza drugs amantadine and rimantadine (Fig 1.5), development of new agents that inhibit this early step in virus replication is still of great interest. Compound *norbornylamine (BL-1743)* (Fig 1.10) is an example of the results obtained so far in this search for M2-inhibitors. BL-1743 was identified after a highly specific high throughout screening for antiviral with profiles similar to that of amantadine (150). This new compound is a spirene-containing drug shown to reversibly inhibit the M2 ion channel of influenza A virus possibly by binding to the open pore of the channel or by altering the conformation of the protein allosterically (262). Discouraging results were found when, after analyzing BL-1743-resistant variants, it was shown that the majority were also amantadine resistant. However, one BL-1743-resistant turned out to be >70-fold more resistant to BL-1743 and only 10-fold more resistant to amantadine than the wild-type virus, suggesting that the interaction with the M2 protein by the two drugs could be different (262).
Figure 1.10 Chemical structures of novel compounds targeting viral uncoating. A) BL-1743, and B) Bafilomycin A1.
A more promising drug candidate targeting the step of viral uncoating is bafilomycin A1 (BFLA1) (Fig 1.10), a macrolide antibiotic with a 16-membered lactone ring identified in 1988 as an extremely potent inhibitor of the vacuolar ATPases (27). Because it is a vacuolar proton ATPase (v-[H⁺]ATPase) that allows for the acidification of endosomes, the ability of bafilomycin A1 to selectively inhibit that enzyme was tested. As a result, replication influenza A and B viruses including H7N3 strains from wild ducks and turkeys, was effectively inhibited in vitro when added before or at the time of virus inoculation (81, 196). Although further in vivo studies are still necessary, bafilomycin A1 seems to efficiently prevent the v-[H⁺]ATPase from pumping protons to the endosome interior at the expense of ATP hydrolysis, thus inhibiting influenza virus uncoating (196).

1.5 Inhibitors of viral RNA synthesis

1.5.1 RNA transcription

The process of influenza viral RNA transcription is one of the most attractive steps of viral replication targeted for drug development. Its distinctive way of initiation by the so called “cap-snatching” process is of particular interest. The first selective inhibitor identified for influenza virus transcription was flutimide (Fig 1.11), a substituted 2,6-diketopiperazine (257). This fungal metabolite was reported to selectively inhibit the cap-dependent transcriptase of influenza A and B viruses (257). Partial characterization of flutimide using assays that uncoupled the reactions of influenza virus
transcription revealed that neither the initiation nor the elongation of influenza virus mRNA synthesis were affected, but it specifically targeted the cap-dependent endonuclease of the transcriptase at low micromolar concentrations (234). This was further confirmed by transcription reactions primed with different capped, synthetic RNA substrates, resulting in the flutimide-mediated inhibition only of those transcription reactions that were primed with capped primers known to undergo endonucleolytic processing and not those capped substrates representing an intermediate in the transcription reaction which could be directly elongated (234).
Figure 1.11 Chemical structures of inhibitors of influenza viral RNA transcription. A) Flutimide, B) L735,882, C) 2-FDG, and D) T-705.
Sharing the same target is \textbf{L735,882} (Fig 1.11), a 4-substituted 2,4-dioxobutanoic acid that has been suggested to selectively inhibit the cleavage of capped RNAs by the influenza virus endonuclease. The latter characteristic provides L735,882 with the ability to inhibit influenza A and B viruses at very low concentrations in both \textit{in vitro} transcription and replication assays (98, 256). Moreover, this compound was tested \textit{in vivo} by infecting the upper respiratory tract of mice, resulting in significant reduction of influenza virus titers (98).

Also under study is the nucleoside analog \textbf{2'-deoxy-2'-fluoroguanosine (2-FDG)} (Fig 1.11), which after being phosphorylated by cellular enzymes is capable of inhibiting influenza A and B transcriptase activity by targeting the active site of the polymerase subunit PB1 (255). Specificity of 2-FDG for this viral enzyme was evaluated and confirmed by different means, including time-of-drug-addition assays, RNA hybridization studies, kinetic studies in the absence of GTP, as well as \textit{in vitro} vRNA synthesis with purified vRNP complexes (255). Moreover, compound 2-FDG has been tested \textit{in vivo}, rendering promising results after reducing pulmonary influenza A and B virus titers in mice (254).

Compound \textbf{T-705} (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) (Fig 1.11) has been shown to have potent inhibitory activity against influenza A, B, and C viruses both \textit{in vitro} (MDCK cells cultures) and \textit{in vivo} (infected mice) (77). Studies for the characterization of this compound found that T-705 can be converted to T-705RMP and T-705RTP by cellular kinases, with the latter being recognized by the influenza virus polymerase as a natural purine nucleotide while the same does not apply for host cell
enzymes which can discriminate all three molecules from the natural nucleotides (78). Further analysis of these products showed a dose-dependent inhibition of the influenza virus RNA polymerase by T-705RTP, and a very weak inhibition of IMPDH (inosine monophosphate dehydrogenase) by T-705RMP which rules out that one as the primary mechanism of action of T-705 (78). Safety and efficacy of this compound for anti-influenza treatment is currently being tested in Phase I clinical trials in the United States and Japan (Toyama Chemical Co., Ltd.)

Keeping in mind the transcription of influenza virus RNA as the target, an alternative approach has been the utilization of *antisense oligonucleotides* (Fig 1.12). It is known that oligonucleotides directed against a gene or mRNA can block transcription or translation through sequence-specific hybridization with targeted genetic segments (114). Based on this, studies testing the ability of this approach to inhibit influenza virus replication have been performed with the design of an antisense oligonucleotide targeted at the PB2 genome (3, 114, 178). In order to obtain higher stability as well as bioavailability *in vivo*, phosphorothioate, a more stable oligonucleotide derivative, was encapsulated using liposomes as delivery vehicle. This liposomally encapsulated antisense phosphorothioate oligonucleotide (S-ODN) was synthesized as a stable chimera formation of DNA and RNA with a dumbbell structure on both ends of the nucleotide displaying cytosine and alkyl loops (3). The oligonucleotide, complementary to sequences surrounding the translation initiation codon (PB2-AUG) of the viral PB2 gene of the influenza A virus RNA polymerases, effectively inhibited influenza virus infection.
of MDCK cell monolayers (3). This highly sequence-specific inhibitory effect was also seen in vivo when S-ODN-PB2-AUG was administered intravenously in mice (178).

1.5.2 Synthesis of nucleic acids

An indirect approach for the inhibition of viral infection is to target the enzymatic activity of inosine monophosphate dehydrogenase (IMPDH), thus reducing the intracellular concentrations of GTP necessary for the synthesis of nucleic acids. This is one of the suggested mechanisms of action of the broad spectrum antiviral drug ribavirin (ribavirin-5'-monophosphate) (Fig 1.13) (82). It has been documented that the inhibition of influenza virus infection by ribavirin starts progressively with the reduction in intracellular GTP with 25 µM concentrations of the drug, while further increase in the amounts (100 µM) reached up to 95% inhibition (287). The latter phenomenon has been proposed to be due to the concerted activity with two other virus-specific mechanisms (i.e. inhibition of 5' -cap formation of mRNAs and inhibition of virus-coded RNA polymerases necessary to prime and elongate viral mRNAs). Ribavirin has been reported to inhibit influenza A and B virus infection both in vitro and in vivo (82). Although not approved by the FDA (Food and Drug Administration) for anti-influenza treatment, this drug has been tested in clinical settings as an aerosol (104, 215) and intravenously (113) with positive results.
**Figure 1.12 Representation of antisense oligonucleotide targeted at the influenza virus PB2 gene.** Phosphorothioate antisense DNA for sequences surrounding PB2-AUG paired with sense RNA and connected by CC-R-CCs (Capital letters: DNA, small letters: RNA, R: alkyl loop structure).
Figure 1.13 Chemical structures of compounds that target the activity of IMPDH.

A) Ribavirin, B) T-CONH2, and C) LY217896.
Ribavirin-5’-monophosphate is actually a pro-drug that gets converted to its 5’-derivatives by cellular enzymes, with its major metabolite being ribavirin-5’-triphosphate (82). Another metabolic product of ribavirin, *1,2,4 triazole carboxamide (T-CONH2)* (Fig 1.13), was tested alone in MDCK cells as well as in mice resulting in comparable anti-influenza activity (228). Moreover, the inherent toxicity of this kind of inhibitor seems to be reduced when T-CONH2 was administered orally to infected mice (228).

Also thought to exert its anti-influenza activity by inhibition of inosine monophosphate dehydrogenase (IMPDH) after being metabolized to the monophosphate form is *1,3,4-Thiadiazol-2-ylcyanamide (LY217896)* (Fig 1.13) (20). Compound LY217896 inhibited the *in vitro* replication of different influenza A and B viruses, effect that was found only after its pre-incubation with the cells before virus inoculation which confirmed that previous intracellular metabolism is required for its antiviral activity (112). LY217896 has also proven to be effective *in vivo* after the observed protection of mice from infection with a lethal dose of influenza A or B virus (48). Its good solubility allowed for administration not only by intraperitoneal injection and aerolization, but in drinking water and oral gavage (48). Results from these studies and additional ones in ferrets suggested that treatment with LY217896 can be delayed for up to 96h after infection, highlighting the potential of this drug for influenza therapy (48).
1.6 Inhibitors of viral progeny release

Novel compounds are under study that target the influenza virus neuraminidase (NA) with the goal of widening the availability of treatments that, although sharing the same target, might be useful if failure of the current ones were to occur. Among these new NA-inhibitor candidates is *peramivir (RWJ-270201)* (Fig 1.14), a cyclopentane derivative product of structure-based drug design (6). Potency of this compound has been thoroughly tested *in vitro*, leading to the studies that leaded to the proposal that peramivir may differ in its antiviral activity when compared to oseltamivir and zanamivir (92). The latter supposition was the result of cross-resistance experiments, which showed that various oseltamivir- and zanamivir-resistant influenza A and B variants retain susceptibility to peramivir (92). Furthermore, various *in vivo* studies using mice as the animal model were performed; ones administered the drug orally to successfully treat lethal H5N1 and H9N2 virus infection, and others used intramuscular injection to prevent lethality in H3N2 and H1N1 influenza infections (11, 87). Moreover, peramivir is currently being tested in humans: (i) a Phase I clinical trial is underway in the United States to evaluate the safety and tolerability of the drug after single and multiple doses (19), (ii) a Phase II multicenter trial is studying the safety and effectiveness of intramuscular injection of peramivir in the treatment of uncomplicated acute influenza (19), (iii) another Phase II clinical study is testing the applicability of peramivir administered intravenously for the prevention of life threatening influenza strains, including H5N1 avian influenza (18). These and future results will guarantee the safety and effectiveness of this promising drug candidate for its use in influenza treatment.
Figure 1.14 Chemical structures of novel NA inhibitors currently under study. A) Peramivir, B) A-315675, and C) Siastatin B.
Also product of structure-based inhibitor design is compound A-315675 (Fig 1.14), a novel tri-substituted pyrrolidine carboxylic acid with potent NA-inhibitory activity in cell culture against strains of influenza A and B viruses (96). Characterization of this compound has included the development of viral resistance as well as cross-resistance studies. Serial passage of influenza A/N9 virus strains in the presence of increasing concentrations of A-325675 resulted in the emergence of the E119D mutation in NA followed by two mutations in the HA a few passages later, same mutations identified in the presence of oseltamivir (179). However, various oseltamivir-resistant and zanamivir-resistant influenza A strains, as well as an oseltamivir-resistant influenza B variant retained susceptibility to A-325675, suggesting minimal cross-resistance between these NA inhibitors in vitro (177, 179). These results suggest possible differences in the mechanism of action of A-325675 when compared to the approved drugs, which makes it an even more interesting candidate if resistance to the latter were to emerge (177).

Finally, a different kind of molecule, 6-acetamido-3-piperidinecarboxylate (Siastatin B) (Fig 1.14) has been characterized resulting to be effective in the inhibition of influenza NA activity (143). This compound has been described as a broad spectrum sialidase inhibitor, property that could be explained by the similarity of the charge distribution in the zwitterionic structure to the putative intermediate in the NA-catalyzed reaction (143). Further characterization of Siastatin B needs to be done, either for its
future use as NA-inhibitor or to serve as a starting point for the design of improved derivative molecules.

1.7 Additional agents with potential anti-influenza therapeutic effect

1.7.1 Immune system stimulation

Exogenous Interferon (PEGylated Interferon-α)

Given its ability to combat virus infection as part of the immune system, administration of exogenous interferon (IFN) was tested by Phillpotts et al. for the prophylactic treatment of influenza (201). Discouraging results were obtained by this group after the intranasal administration of purified lymphoblastoid IFN (HuIFN-α) one day before virus challenge (201). Outcomes like this have possibly underestimated the potential of IFN as an alternative for influenza treatment. However, recent advances in antiviral research have uncovered improved options for the delivery of IFN that has made it usable for human treatments. One of them is pegylation, the covalent attachment of an inert water-soluble polymer of polyethylene glycol (PEG) to the IFN molecule (62). PEGylated interferon-alpha combined with ribavirin not only has been proven to improve the antiviral effect of this drug, but it has become the first option for treatment of hepatitis C (62). Therefore, investigational studies are underway to test the anti-influenza potential of IFN, including a Phase I clinical trial conducted by the National Institute of
Allergy and Infectious Diseases (NIAID) that evaluates if IFN added to a specific influenza vaccine helps the immune system more efficiently than the vaccine alone (190).

**Double-stranded RNA (poly(I)-poly(C))**

The anti-influenza prophylactic and therapeutic efficacies of poly(I)-poly(C) as a known IFN inducer have been evaluated in mice after its intranasal administration before virus inoculation (284, 285). For this, synthetic double stranded polyriboinosinic-polylribocytidylic acid stabilized with polylysine and carboxymethylcellulose (Poly-ICLC) was encapsulated in cationic liposomes enhancing the immunomodulating activity of this molecule (284). Results from these studies suggested that intranasal Poly-ICLC provides protection against mortality in mice for influenza virus infection (284, 285). The induction of interferons, cytokines, and chemokines triggered by Poly-ICLC is currently under study for its use in humans. Interestingly this approach is undergoing Phase I clinical trials for the examination of the safety and effectiveness in preventing or reducing the severity of infections not only from influenza, but also from other viruses acquired through the nose, mouth, and lungs (189).

**1.7.2 RNA interfering**

*Small interfering RNAs (siRNAs)* are 21-25 nucleotide RNA molecules that act post-transcriptionally inducing the sequence-specific degradation of homologous mRNA by cellular enzymes (202). A variety of research studies have previously reported the potential of this approach for combating viruses and other human pathogens (58). This
led Ge et al. (79) and Tompkins et al. (259) to test siRNAs for the inhibition of influenza A virus \textit{in vivo}. Their results, which were confirmed not to be due to induction of the IFN system, showed the ability of siRNAs specific for conserved regions of the virus nucleoprotein (NP) or acidic polymerase (PA) genes to prevent and treat influenza virus infection in mice. Reduced lung virus titers were observed even in mice infected with highly pathogenic avian influenza A viruses of the H5 and H7 subtypes. Although application of siRNAs for prophylaxis and therapy of influenza virus in humans will require further development of compatible delivery systems, the effectiveness of this alternative is highly promising (15).
CHAPTER II

Screening of small molecule library for the identification of potential anti-influenza virus compounds

Introduction

Prophylactic and therapeutic antiviral drugs for influenza are available as an adjunct to vaccination. However, their effectiveness is already being limited because of the rapid emergence of drug resistant isolates due to the vast use of these drugs and the recombination potential of the viral genome. What is more, because there is no palpable resource to control an influenza pandemic, the use of vaccines and antiviral drugs together with quarantine and masks will be indispensable in order to mitigate the socio-economic impact. However, even if the production of a vaccine started the same day that the pandemic is declared it would take from four to six months to produce the first doses (280). Hence, the appropriate use of antiviral agents would be the only feasible option. Influenza H5N1 strains, the immediate threat nowadays, are sensitive to the NA inhibitors but not to the adamantane derivatives (134). Despite the good news, issues like the availability of drugs as well as the potential for the pandemic strain to acquire NA resistance are still pending. On one hand, the DHHS is working with the private-sector to increase supplies of antivirals for the Strategic National Stockpile (SNS), which by the end of 2005 contained 2.26 million treatment regimens of oseltamivir and 84,000 of
zanamivir for a country with approximately 295 million people (41). On the other hand, the frequency of emergence of resistance during NA treatment of patients with H5N1 infection is still uncertain; however, the isolation of oseltamivir-resistant influenza A (H5N1) variants has been already reported (61).

Given the obvious need for additional antiviral agents as well as their availability and surge capacity, our study intended to identify new compounds with potential anti-influenza activity. This was done by screening a library of 34,000 organic small molecules by means of a cell-based system that assayed the inhibition of virus-induced cell death. This HTS assay was developed under stringent standards for high sensitivity, reproducibility, and accuracy. As a result, a group of primary hits was identified that fulfilled the conditions established of cellular protection from virus cytopathic effects with low or no inherent cytotoxicity at the concentration tested. A second screening of this group of compounds narrowed down the number of hits by eliminating false-positives, typical of large high-throughput screens (8), and confirming those with antiviral activity. More thorough evaluation of the primary hits was done by determination of their 50% inhibitory (IC$_{50}$) and 50% cytotoxicity concentrations (CC$_{50}$). Analysis and comparison of these values allowed for the selection “lead” compounds, i.e., those with the most promising therapeutic index (TI). Detailed description of the methodologies used, as well as the findings obtained during this study are included in this chapter.
Materials and methods

Cell lines. Madin-Darby canine kidney cells (MDCK) were routinely passaged in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml) and maintained at 37°C, 5% CO₂. Influenza A detecting cell lines IAV-Luciferase (ELVIRA®FloA) and IAV-GFP were obtained from Diagnostic HYBRIDS, Inc (DIAGNOSTIC HYBRIDS; Athens, OH). In these cell lines the influenza A virus NP protein was substituted by the firefly luciferase or enhanced green fluorescent protein (GFP) open reading frames in the negative sense, conserving the 5' and 3' untranslated regions, and cloned between the human RNA polymerase promoter and murine RNA polymerase terminator. This allows the viral polymerase to recognize and replicate the RNA present in FluA luc or FluA GFP-expressing cells (167). Both reporter cell lines were cultured in DMEM containing 10% FBS, 50 µg/ml Hygromycin and penicillin-streptomycin (100 U/ml). Right before infection, growth medium was replaced by RMO3T, a reduced-serum medium containing penicillin-streptomycin (100 U/ml) and 0.5 µg/ml TPCK-Trypsin (Diagnostic HYBRIDS, Inc., Athens, OH).

Viruses. Laboratory-adapted influenza strain A/WSN/33 was obtained from Dr. P. Christopher Roberts (Virginia Tech College of Veterinary Medicine, Blacksburg, VA). This virus had been previously propagated by infecting 10-day-old embryonated chicken
eggs. Allantoic fluid containing virus was harvested 48h post-inoculation. Titer of virus stock was determined by plaque assay on MDCK cell monolayers.

**Drugs.** A library of 34,000 compounds was manufactured by ChemBridge Corporation (San Diego, California), and supplied by the Small Molecule Screening Core at Cleveland Clinic (Cleveland, Ohio). Aliquots of primary hits used for further rounds of screening were obtained from the same core. One milligram aliquots of compounds named QMV-13, QMV-13A, QMV-13B, QMV-15, QMV-15A, QMV-15B, and QMV-15C, were purchased directly from ChemBridge Corporation (San Diego, California). Lyophilized powder was dissolved in DMSO at a concentration of 10mM and stored at -20°C. Oseltamivir phosphate was provided by Roche (Hoffmann-La Roche, Switzerland) as a lyophilized powder and was dissolved in sterile distilled water at a concentration of 10mM. Aliquots of the stock solution were kept at -20°C and diluted in cell culture media right before use.
Fig. 2.1 High throughput screening (HTS) system used to identify potential anti-influenza compounds. (A) Schema of the plate format used. (B) HTS method performed to detect compounds with anti-influenza virus activity.
High throughput screening (HTS). 2.5x10^4 MDCK cells per well were seeded in 96-well plates in 100ul of culture media and incubated overnight at 37°C, 5% CO₂. Cell monolayers were washed with PBS and placed in 50ul of DMEM medium containing penicillin-streptomycin (100 U/ml) and 1.5 µg/ml of L-1-(tosyl-amido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corp., Lakewood, NJ). Single aliquots (0.2ul) of the compounds (dissolved in DMSO) were added by replicators in 80 wells as well as 10 µM of oseltamivir phosphate (two wells) as a control (Fig. 2.1). After 2 hours of incubation at 37°C, 5% CO₂, 50ul of diluted influenza virus A/WSN/33 (H1N1) were added at a multiplicity of infection (MOI) of 0.005 IU/cell, leaving 3 wells for no virus, no drug control. 48h after infection, cell protection assays were performed as described below (Fig 2.1). Percentage of cell survival was calculated relative to the average of no virus wells (100%) per plate.

Cell protection assay (MTT Assay). After incubation of cell monolayers with the compounds for 48 hours, a colorimetric MTT (tetrazolium) assay (182) was performed in order to measure cell survival. For this, supernatant was removed from each well and 100ul of 0.4mg/ml methylthiazolyldiphenyl-tetrazolium bromide solution (MTT, Sigma-Aldrich, St. Louis, MO) were added followed by 4 hours incubation at 37°C, 5% CO₂. After this, the solution was removed (without disturbing the crystals) and 100ul of DMSO were added per well. After a few minutes at room temperature the plates were read at 570nm in a microplate reader (Wallac 1420 Victor³V, Perkin Elmer, Waltham, MA).
**Drug susceptibility assays.** Fifty percent inhibitory concentrations (IC$_{50}$) of primary hits were determined using two different but complementary methods:

(i) The same method as for the HTS, where serial dilutions of the compounds were added 2h before the addition of the virus. Infection of the cells was done at a MOI of 0.005 IU/cell for 48h. After this, supernatants were removed and colorimetric MTT (tetrazolium) assays were done as described above.

(ii) Measurement of luciferase activity in ELVIRA®FluA cells. About 24h before the experiment $5\times10^4$ cells/well were plated onto white opaque-walled 96-well plates (BD Biosciences, Bedford, MA) pre-treated with 40 µg/ml human natural fibronectin (BD Biosciences, Bedford, MA). Complete medium was replaced by RMO3T right before the addition of compounds. Cells were infected at a MOI of 0.05 IU/cell after a 2h pre-incubation with the drugs, followed by the determination of luciferase activity 24h post-infection. This was done by removing the supernatants from the wells, followed by lysis of the cell monolayers (1X Cell culture lysis reagent, Promega Corporation, Madison, WI) and addition of 100ul/well of Luciferase Reagent (Promega Corporation, Madison, WI) after 10 minutes of shaking. Readings of the light intensity (RLU/sec) were done with a microplate reader (Wallac 1420 Victor$^{3}$V, Perkin Elmer, Waltham, MA).

Independently of the method used, antiviral activity of the compounds was defined as the 50% inhibitory concentration (IC$_{50}$) calculated relative to controls using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).
**Drug cytotoxicity.** Cytotoxicity of the primary hits was evaluated by incubating serial dilutions of each compound with MDCK cell monolayers. After 2h incubation, supernatants were removed and MTT assays (colorimetric determination of cell survival described above) were performed. The concentration of compound with 50% cytotoxic effect (CC$_{50}$) was calculated relative to “no drug” control wells using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).

**Results**

2.1.1 **Cell-based colorimetric system is suitable for high throughput screening (HTS)**

The screening assay developed consisted in the quantification of Madin Darby canine kidney (MDCK) cells viability after influenza A/WSN/33 (H1N1) virus infection by means of a tetrazolium-based colorimetric assay (MTT) (Fig. 2.1). In order to assure the performance of a high-quality HTS we first evaluated the reliability of this system by determining its Z’ value (Fig. 2.2) (290). This common measure of assay performance and consistency takes into account both the “signal-to-background” ratio (signal dynamic range) and “signal-to-noise” ratio (variation) (176). A HTS assay can be considered excellent when its Z’ value scores between 0.5 and 1, with 1 being “perfect” (290). This determination was done by assaying three 96-well plates containing positive and negative controls on two separate days (well-to-well and day-to-day variation). Using healthy cells as positive control and infected cells as negative control, the HTS assay selected for this
screening showed $Z'$ values of 0.72 (day 1) and 0.59 (day 2) resulting in an average of 0.655, indicating assay reliability (Fig. 2.2). The difference in the $Z'$ values obtained between days 1 and 2 reflects the actual purpose of this validation of mimicking the conditions during the HTS and their possible variations. In this particular case, although coming from the same batch, the cells used for each determination had undergone different number of passages in culture, which explains their differences in metabolism levels. In order to avoid this variable, MDCK cell cultures were not passaged more than 12 times during the performance of the HTS.
Figure 2.2 Z’ Value Determination. (A) Mathematical derivation of Z’-factor ($\sigma$ = standard deviation, $\mu$ = mean) (290). (B) This figure shows the results of an average of three Z’ tests performed simultaneously in one day. The wells of three 96-well plates were seeded with MDCK cells ($2.5 \times 10^4$/well). The next day, cells were washed with PBS and replenished with DMEM containing 1.5 µg/ml of TPCK/Trypsin. Diluted virus (0.005 MOI) was added to 48 wells of each plate and incubated for 48h, followed by performance of MTT assays and determination of cell survival. The resulting Z’ value for this experiment was 0.720. The same procedure was followed on a different day and the average of the two determinations was 0.655 (>0.5).
A

\[ Z' = 1 - \frac{3\sigma_{\text{pos}} + 3\sigma_{\text{neg}}}{\mu_{\text{pos}} - \mu_{\text{neg}}} \]

B

[Graph showing absorbance (570nm) vs. well number]

- **Uninfected**
- **Infected**

*Z' Value = 0.72*
2.1.2 Identification of primary hits

Once the system was validated by the compliance with $Z'$ factor standards for high throughput screening assays we started the search for possible “hits”. The chemical library screened in this study consisted of 34,000 organic small molecules manufactured by ChemBridge Corporation (San Diego, California) available at the Small Molecule Screening Core of the Cleveland Clinic (Cleveland, Ohio). Structure and purity (>95%) of these molecules were validated by nuclear magnetic resonance (NMR), and their molecular weight was reported to range between 250 and 550 g/mol. This small molecule library has been used by different groups for the identification of potential therapeutic agents with potential properties against widely diverse targets, such as the tumor suppressor protein 53 (p53) (94, 146, 147), interferon-induced ribonuclease RNase L (252), human parainfluenza virus type 3 (168), and human immunodeficiency virus type 1 (HIV-1) (132, 133).

The format and protocol designed for this particular HTS allowed for the screening of 80 compounds per 96-well plate each of which had a final concentration of 20-40 μM, depending on their molecular weight. In order to limit our search to only highly active anti-influenza compounds, our hit threshold (290) was set to those compounds with antiviral activity similar to that of the no virus control drug (100% cell survival). According to this standard, the primary screening of the small molecule library resulted in a set of 330 “primary hits” (0.97%), which were re-screened using the same system. The latter allowed for the confirmation of 24 hits (0.07%) with anti-influenza activity.
The antiviral effect of all 24 hits was further evaluated by pre-treatment of MDCK cells with serial dilutions of the compounds, followed by inoculation of virus (A/WSN/33) and performance of cell protection assays (MTT) 48h after infection. Based on these results, 12 compounds that provided increasing cell protection starting from relatively low concentrations were selected as potential “leads” (i.e., QMV-1, QMV-4, QMV-5, QMV-8, QMV-9, QMV-13, QMV-15, QMV-16, QMV-18, QMV-19, QMV-21, and QMV-24) (Fig. 2.3).
Figure 2.3 Determination of drug susceptibility of 24 selected hits by cell protection assay. After 48h incubation, MTT assays were performed and absorbance was determined (570nm) in a microplate reader. Percentages (%) of cell survival were calculated relative to “no virus” controls. (A) Group of 12 small molecules selected for further study due to their lower IC₅₀s and less or no additional cytotoxic effect at inhibitory concentrations. (B) Compounds excluded from the list of selected hits because of their relatively high IC₅₀ values.
2.1.3 QMV-13 and QMV-15 display the most promising Therapeutic Indices (TI).

Uninfected MDCK cell monolayers were incubated for 48h with increasing concentrations of the 12 pre-selected hits to determine the concentration at which they become toxic to 50% of cells (CC\textsubscript{50}). Comparison of the latter values relative to fifty percent inhibitory concentrations (IC\textsubscript{50}) previously determined, provided us with the therapeutic index (TI) of each compound (Table 2.1). This value (CC\textsubscript{50}/ IC\textsubscript{50}), is a common indicative of the selectivity of a drug and consequently its effectiveness and usability (222). Therefore, based on their low cytotoxicity at the highest concentration tested (50 µM) and high TI compared to a known antiviral drug (i.e. oseltamivir phosphate), QMV-13 and QMV-15 were chosen as “lead compounds” for further characterization.
Table 2.1 IC$_{50}$, CC$_{50}$ and TI of 12 pre-selected hits. Evaluation of Therapeutic Index (TI) of the 12 pre-selected hits after determination of their IC$_{50}$ and CC$_{50}$ values by means of cell survival assays (MTT assay). Due to their effective antiviral activity at lower concentrations and their safe cytotoxicity levels, compounds QMV-13 and QMV-15 were selected for further characterization.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Structure</th>
<th>CC$_{50}$ (µM)</th>
<th>IC$_{50}$ (µM)</th>
<th>TI (CC$<em>{50}$/IC$</em>{50}$)</th>
<th>% Cell Protection at (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oseltamivir</td>
<td><img src="oseltamivir.png" alt="Structure" /></td>
<td>&gt;50</td>
<td>6.19</td>
<td>&gt;8.07</td>
<td>100% (50µM)</td>
</tr>
<tr>
<td>QMV-13</td>
<td><img src="qmv-13.png" alt="Structure" /></td>
<td>&gt;50</td>
<td>5.02</td>
<td>&gt;9.96</td>
<td>100% (10 µM, 50 µM)</td>
</tr>
<tr>
<td>QMV-15</td>
<td><img src="qmv-15.png" alt="Structure" /></td>
<td>&gt;50</td>
<td>6.75</td>
<td>&gt;7.42</td>
<td>98.40% (50 µM)</td>
</tr>
<tr>
<td>QMV-18</td>
<td><img src="qmv-18.png" alt="Structure" /></td>
<td>&gt;50</td>
<td>17.39</td>
<td>&gt;2.88</td>
<td>98.46% (50 µM)</td>
</tr>
<tr>
<td>QMV-19</td>
<td><img src="qmv-19.png" alt="Structure" /></td>
<td>&gt;50</td>
<td>13.50</td>
<td>&gt;3.7</td>
<td>100% (50 µM)</td>
</tr>
<tr>
<td>QMV-4</td>
<td><img src="qmv-4.png" alt="Structure" /></td>
<td>50</td>
<td>3.12</td>
<td>16.05</td>
<td>50.03% (50 µM)</td>
</tr>
<tr>
<td>QMV-9</td>
<td><img src="qmv-9.png" alt="Structure" /></td>
<td>50</td>
<td>2.67</td>
<td>18.71</td>
<td>115% (50 µM)</td>
</tr>
<tr>
<td>QMV-16</td>
<td><img src="qmv-16.png" alt="Structure" /></td>
<td>50</td>
<td>5.14</td>
<td>9.72</td>
<td>85.75% (10 µM)</td>
</tr>
<tr>
<td>Compound</td>
<td>Structure</td>
<td>IC₅₀ (µM)</td>
<td>IC₅₀ (µM)</td>
<td>% Inhibition</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>QMV-24</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>50</td>
<td>7.69</td>
<td>6.5</td>
<td>100% (50 µM)</td>
</tr>
<tr>
<td>QMV-8</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>45</td>
<td>18.52</td>
<td>2.43</td>
<td>77.20% (5 µM)</td>
</tr>
<tr>
<td>QMV-21</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>40</td>
<td>4.97</td>
<td>8.05</td>
<td>91.09% (10 µM)</td>
</tr>
<tr>
<td>QMV-5</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>40</td>
<td>2.81</td>
<td>14.21</td>
<td>97.58% (5 µM)</td>
</tr>
<tr>
<td>QMV-1</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>20</td>
<td>2.59</td>
<td>7.73</td>
<td>76.40% (50 µM)</td>
</tr>
</tbody>
</table>
2.1.4 QMV-13 and QMV-15 structural analogs

To elucidate the minimal pharmacophore for anti-influenza activity of compounds QMV-13 and QMV-15 we obtained structurally related compounds by searching in ChemBridge library databases (Fig. 2.4). Anti-influenza activities of these small molecules, named QMV-13A, QMV-13B, QMV-15A, QMV-15B, and QMV-15C, were assessed using the same methods as for the identification of the lead compounds (MTT assay) (Fig 2.5). Analog QMV-13A showed complete inhibition of virus infection at a very low concentration (1 µM), but also demonstrated to be highly cytotoxic with a drastic drop in cell survival at concentrations higher than 10 µM. On the other hand, compound QMV-15C did not show antiviral activity at any of the concentrations tested (Fig 2.5). In contrast, analogs QMV-13B, QMV-15A, and QMV-15B exhibited significant cell protection from virus-induced CPE, with low cytotoxicity. Correlation between these results and the different chemical structures led us to the identification of functional groups within QMV-13 and QMV-15 that are essential for the preservation of their antiviral properties and safety \textit{in vitro}. Moreover, the potential usefulness of analogs QMV-13B, QMV-15A, and QMV-15B in anti-influenza therapy led us to their inclusion in the group of leads for further characterization.
Figure 2.4 Structural Analogs of QMV-13 and QMV-15. Compounds structurally related to (A) QMV-13, and (B) QMV-15 were identified and obtained from ChemBridge repository using a searchable database (www.Hit2Lead.com). (Red circles indicate structural differences compared to the parental molecule).
B

QMV-15

QMV-15A

QMV-15B

QMV-15C
Figure 2.5 Potential anti-influenza virus activity of structural analogs of QMV-13 and QMV-15. Antiviral effects, as well as cytotoxicity of these analogs were determined in parallel with their parental molecule (A) QMV-13, and (B) QMV-15 by cell survival assays (MTT assay). IC$_{50}$ and CC$_{50}$ values were calculated using Prism version 5.00 for Windows (GraphPad Software, San Diego, California), and the results obtained were used to calculate the Therapeutic Indexes (TI) (CC$_{50}$/ IC$_{50}$).
**A**

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$</th>
<th>CC$_{50}$</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMV-13</td>
<td>6.50</td>
<td>50</td>
<td>7.69</td>
</tr>
<tr>
<td>QMV-13A</td>
<td>0.70</td>
<td>11.45</td>
<td>16.4</td>
</tr>
<tr>
<td>QMV-13B</td>
<td>1.44</td>
<td>50</td>
<td>34.7</td>
</tr>
</tbody>
</table>

% Cell Survival (Relative to no virus controls)

**B**

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$</th>
<th>CC$_{50}$</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMV-15</td>
<td>6.60</td>
<td>50</td>
<td>7.57</td>
</tr>
<tr>
<td>QMV-15A</td>
<td>4.90</td>
<td>50</td>
<td>10.2</td>
</tr>
<tr>
<td>QMV-15B</td>
<td>5.50</td>
<td>50</td>
<td>9.10</td>
</tr>
<tr>
<td>QMV-15C</td>
<td>&gt;50</td>
<td>50</td>
<td>~</td>
</tr>
</tbody>
</table>

% Cell Survival (Relative to no virus controls)


Discussion

In this study, we describe a cell-based HTS assay that can be used not only to identify potential influenza inhibitors but also to avoid compounds with undesirable toxicity as well as to simultaneously test a variety of drug mechanisms without the narrowness of target specificity common with other HTS influenza assays. The suitability of the assay for HTS was confirmed with the calculation of Z' values >0.5 (0.655), which validated the assay parameters chosen (290). This proved the reproducibility of the system, confirming our results while allowing for multiple days of screening and comparison of data quality across screens (272). Various factors of the assay, such as virus strain and cell line selected, were fundamental in the design of this successful HTS system. Influenza A/WSN/33 (H1N1) virus is a well characterized laboratory-adapted strain that was selected by mouse brain passage of the A/WS/33 (H1N1) virus, a descendant of the virus responsible for the 1918 pandemic (85). On one hand, in the search for a stringent system that would result in the identification of highly potent influenza inhibitors, we decided on this neurovirulent strain that has been found to be less sensitive to drug inhibition in vitro than H3N2 and other strains (192, 232, 239). On the other hand, A/WSN/33 displays highly cytolytic activity, property that adds to the desired stringency of our system and facilitates the readout during cell protection assays (MTT assays) by enhancing the difference between “cell survival” and “cell death” signals. With that goal in mind, and taking into consideration that most antiviral compounds
exhibit variable activity depending on the amount of virus present (232), we selected a MOI that induced maximal CPE in 48h (0.005 IU/cell).

Another parameter crucial for the utilization of our system as HTS was the choice of cell type. Although other cell lines such as primary chick embryo, chick kidney, calf kidney, Vero, mink lung, and human respiratory epithelial cells may be used, Madin Darby Canine Kidney (MDCK) cells are the preferred option for in vitro antiviral assays (232). This cell line not only expresses sufficient amounts of both α-(2,6) or α-(2,3)-linked sialic acids to allow the growth of a wide variety of virus strains (99, 192), but has also been shown to tolerate relatively high concentrations of DMSO in assay media (up to 1% DMSO in serum-containing media) (127). To corroborate this, we tested the effect of various concentrations of DMSO on MDCK cell monolayers by MTT assay, resulting in near to 100% cell survival at more than 2% DMSO after 48h incubation (data not shown). Moreover, potential interference of DMSO in the assay throughout the screening process was assessed for every plate with the addition of 0.2ul of DMSO to three wells, volume that is equivalent to that one of the compounds. The selection of influenza A/WSN/33 (H1N1) and MDCK cells, together with optimal conditions like MOI and incubation times (pre-incubation with drug and incubation with the virus), made of this cell-based assay a sensitive and reliable system for HTS.

Screening of the chemical library of small molecules was performed in two stages. The first stage or primary screening consisted in the evaluation of a single concentration of all 34,000 compounds for antiviral activity. According to our standards, those compounds with antiviral activity similar to that of the control drug (100% cell
survival) were defined as “primary hits”, resulting in a set of 330 compounds which represented 0.97% (Fig 2.6) of the small molecules tested. In the second stage of the screening process, the primary hits were retested and 24 of them were confirmed as active “hits”, with a success rate of 0.07%. Initial high rates like the one we obtained after the first stage of our screening process are common and have been described in previous studies as the result of a high degree of variability inherent in assays comprising large numbers of samples (8). False-positives and false-negatives are often present in HTS assays as a consequence of instrumental and human-associated errors such as degradation or low purity of compounds, inconsistent plate replication, and evaporation of compound solutions, among many others (8, 268). Such drawbacks were drastically reduced with a more thorough round of drug susceptibility determinations done individually with the 24 pre-selected hits. From this results, 12 compounds displaying sustained antiviral activity (i.e. cell survival) at the highest concentration tested (50 \( \mu M \)) were selected for further evaluation.

In order to narrow down our search identifying those hits with the most promising antiviral properties, we turned to their therapeutic indices (TI). This widely recognized ranking measure of drug effectiveness is the ratio between the concentration of compound required for efficacy vs. the one at which it becomes toxic (CC_{50} / IC_{50}) (200, 222). Compounds QMV-13 and QMV-15 joined our drug control (oseltamivir) at the top of our ranking list due to their >50 \( \mu M \) cytotoxicity values in MDCK cells. An endpoint concentration could not be reached during CC_{50} determinations of these two compounds due to the cytotoxicity of their solvent (DMSO) at >2% in MDCK cells. An attempt to
use a different solvent less toxic to the cells was not feasible due to the reduced solubility of QMV-13 and QMV-15. Although not ideal, this characteristic is very common among extensive chemical libraries for HTS where high lipophilicity of compounds is handled by dissolving them in dimethyl sulfoxide (DMSO) as stock solutions, thus making it possible to test even very insoluble drugs (162).
Figure 2.6 Summary of HTS of 34,000 small molecules library for anti-influenza compounds. 24 Hits were confirmed after re-testing the 330 primary hits using the same system (MTT assay). From those, 12 hits displaying lower IC\textsubscript{50} and higher CC\textsubscript{50} values were tested manually, leading to the selection of QMV-13 and QMV-15 due to their promising Therapeutic Indexes (TI).
Physicochemical properties like solubility, membrane permeability, and oral bioavailability, are aspects of compound’s profiles that need to be optimized for them to be considered potential drug candidates (211). A set of parameters statistically shown to improve probability of success in drug development was described by Lipinski with his known “Rule of 5” (162). According to this rule, absorption and bioavailability are likely to be higher if (162, 211):

(i) Molecular weight <500  
(ii) Log P <5  
(iii) H-bond donors <5 (expressed as the sum of OHs and NHs)  
(iv) Sum of N and O (H-bond acceptors) <10  
(v) With substrates for biological transporters as an exception to the rule.

Compound optimization involves reiterative medicinal chemistry with the development and synthesis of chemical analogs to find a compound with the described characteristics. As a first step into this lead optimization phase, we tested a few structural analogs of compounds QMV-13 and QMV-15 in vitro, using the same system as for the HTS (Fig 2.5). From the results obtained, the appearance of high toxicity (QMV-13A) and complete loss of antiviral properties (QMV-15C) due to known changes in the chemical structure of these compounds provided us with initial information about their pharmacophore (Fig 2.7). Further combinatorial chemistry and SAR (Structure-Activity Relationship) studies would provide us with more detailed insights about their active molecular framework, a crucial instrument in the optimization of their antiviral properties of the lead compounds as well as their physicochemical properties (80, 122).
Figure 2.7 Comparison among antiviral properties of the parental molecules (A) QMV-13 and (B) QMV-15 with their analogs. This figure shows the effect of the chemical structures upon the therapeutic indexes (TI) of the analogs. Red arrows indicate those structural modifications with negative effects on the effectiveness and safety of the analogs with respect to their parental molecule, while blue arrows indicate either sustained or improved properties. IC$_{50}$ and CC$_{50}$ values used in these graphs were determined by cell survival assays (see Fig 2.7).
A

![Graph showing IC50 and CC50 for QMV13A and QMV13B](image)

B

![Graph showing IC50 and CC50 for QMV15A, QMV15B, and QMV15C](image)
Replacing the 2-thiofuryl substituent of QMV-13 with 2-furyl and desaturating the C-N bond improved its inhibitory concentration ten-fold but increased its toxicity five-fold (QMV-13A) (Fig 2.7). On the other hand, replacing it with phenyl and maintaining the C–N bond saturation yielded QMV-13B with a five-fold improved activity without increasing its cytotoxicity (Fig 2.7). It appears that having the N-C-N bonds in a saturated state is critical for keeping the compound’s cytotoxicity low. For QMV-15, it seems that the methyl substituent in para-position on the amino-phenyl ring is important for its anti-viral activity as it is increases >10-fold when comparing QMV-15A to QMV-15C (Fig 2.7). Moreover, based on the same two structures, it could be that the atomic space volume of the methyl and bromine substituents, which are considerably bigger than fluorine, may play an important role in the steric interaction between the chemical compound and its target. On the other hand, conjugating the aldehyde group with semicarbazide or semithiocarbazide improves only minimally the antiviral properties of the compounds.

In conclusion, the cell-based HTS system validated in this study demonstrated to be useful for the identification of compounds inhibiting replication-competent influenza virus at any stage of its life cycle, simultaneously excluding those small molecules with inherent cytotoxicity. Using this system, we screened a library of 34,000 small molecules and identified two potential anti-influenza compounds with novel structural scaffolds different from those already approved for antiviral therapy. Further analysis of a group of structural analogs not only suggested the pharmacophore of our leads, but also identified three more compounds with promising antiviral properties.
CHAPTER III

Characterization of anti-influenza properties of compounds with antiviral activity against influenza virus

Introduction

As a response to the deficit of available antiviral drugs for the treatment of influenza as an emerging infectious disease, we screened a large library of small molecules and identified two compounds (QMV-13 and QMV-15) and three of their analogs (QMV-13B, QMV-15A, QMV-15B) as potential candidates for the development of novel anti-influenza treatments. Following this accomplishment, we conducted a series of assays that contributed to the description of their mechanism of action. First, we evaluated their likelihood to succeed as candidates for further optimization and development into investigational drugs. For this, thorough evaluation of their cytotoxicity in a variety of primary and immortalized cell lines was done, followed by the assessment of their antiviral properties. The leads and three of their analogs showed antiviral activity against a powerful laboratory adapted strain of influenza A (A/WSN/33) when tested by means of different methods. Furthermore, replication of a cohort of laboratory-adapted and clinical influenza A and B isolates was quantified in the presence of the lead compounds resulting in low IC$_{50}$ values. Drug susceptibility (IC$_{50}$) determinations against influenza virus strains resistant to currently approved antiviral drugs resulted in their
inhibition in a dose-dependent manner. The next step in the characterization of the lead compounds was to test the specificity of their antiviral activity against other RNA viruses such as YFV, WNV, hPIV3, and HIV, ruling out the possibility of a broad spectrum of action. Time-of-drug-addition assays shed light on the stage of the virus reproductive cycle targeted by the compounds under study. In addition, their effect on viral protein expression was evaluated, as well as on the virus growth dynamics after single and multiple rounds of replication. Finally, serial passages of the virus in the presence of the lead compounds were done aimed to select drug resistant variants that could pinpoint more accurately their target.

In this section we report the promising results from our characterization studies which could certainly guide us toward the development of short term resources as investigational new drugs in case of contingency measures during an influenza pandemic and possibly a future alternative anti-influenza treatment.

**Materials and methods**

**Cell lines.** Madin-Darby canine kidney cells (MDCK) were routinely passaged in Dulbecco modified Eagle medium (DMEM; Cellgro, Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS; Cellgro) and penicillin-streptomycin (100 U/ml) and maintained at 37°C, 5% CO₂. Influenza A detecting cell lines IAV-Luciferase (ELVIRA®FluA) and IAV-GFP were obtained from Diagnostic HYBRIDS, Inc (DIAGNOSTIC HYBRIDS; Athens, OH). In these cell lines the influenza A virus NP
protein was substituted by the luciferase or GFP open reading frames in the negative sense, conserving the 5’ and 3’ untranslated regions, and cloned between the human RNA polymerase promoter and murine RNA polymerase terminator. This allows the viral polymerase to recognize and replicate the RNA present in FluA luc or FluA GFP-expressing cells (167). Both reporter cell lines were cultured in DMEM containing 10% FBS, 50 µg/ml Hygromycin and penicillin-streptomycin (100 U/ml). Right before infection, growth medium was replaced by RMO3T, a reduced-serum medium containing penicillin-streptomycin (100 U/ml) and 0.5 µg/ml TPCK-Trypsin (Diagnostic HYBRIDS, Inc., Athens, OH). Continuous cell lines A549 human lung carcinoma cells, MRC-5 human embryonic lung fibroblasts, NCI-H292 human pulmonary muco-epidermoid carcinoma, WI-38 human embryonic lung fibroblasts, NHFL neonatal human lung, Vero African green monkey kidney cells, and HeLa human cervix adenocarcinoma cells were also obtained from Diagnostic HYBRIDS, Inc (DIAGNOSTIC HYBRIDS; Athens, OH). DMEM containing 2% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml) was used as refeed medium for all the experiments. Astroglioma cells U87.CD4.CXCR4 and U87.CD4.CCR5 were obtained through the AIDS Research and Reference Reagent Program (ARRRP) and maintained in DMEM supplemented with 15% FBS, penicillin-streptomycin (100 U/ml), puromycin, and geneticin. Primary Sprague Dawley Rat hepatocytes used for in vitro toxicology assays were provided by Diagnostic HYBRIDS Life Science Division (DIAGNOSTIC HYBRIDS; Athens, OH).
**Viruses.** Laboratory-adapted influenza strain A/WSN/33 was obtained from Dr. P. Christopher Roberts (Virginia Tech College of Veterinary Medicine, Blacksburg, VA). Recombinant influenza virus A/Udorn/72 was provided by Dr. Andrew Pekosz (Johns Hopkins University, Baltimore, MD). A series of different laboratory-adapted, clinical isolates and drug-resistant influenza A and B viruses (Table 3.1) were obtained from Dr. P. Christopher Roberts (Virginia Tech College of Veterinary Medicine, Blacksburg, VA), Dr. Larisa Gubareva (Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia), Diagnostic HYBRIDS, Inc (Athens, OH), and American Type Culture Collection (ATCC, Manassas, VA). Virus stocks were generated by infecting MDCK cells and 50% tissue culture infectivity dose (TCID<sub>50</sub>) was determined either in MDCK cells as described below or by luciferase expression depending on the nature of the experiments. Laboratory strains of Yellow Fever virus (YFV; strain YFV-17D), and West Nile virus (WNV; strain NY-99) were obtained from the Virus Core at the Cleveland Clinic (Cleveland, Ohio) and the CDC (Centers for Disease Control and Prevention; Atlanta, Georgia), respectively. Human parainfluenza virus 3 (hPIV3; HA-1) was provided by Dr. Amiya Banerjee at the Lerner Research Institute (Cleveland Clinic, Cleveland, Ohio).
Table 3.1 Influenza A and B laboratory-adapted, clinical isolates and drug-resistant viruses used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSN/33 (H1N1)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>WS/33 (H1N1)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Texas/36/91 (H1N1)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>PR/8/34 (H1N1)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>C1d (H1N1)</td>
<td>A</td>
<td>Laboratory Adapted</td>
</tr>
<tr>
<td>HongKong/8/68 (H3N2)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>A/Udorn/72 (H3N2)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Victoria/3/75 (unknown subtype)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Maryland/1/59</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>GL/1739/54</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Russia/69</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>SKCPHL 05V2625 (H3N2)</td>
<td>A</td>
<td>Clinical Isolate</td>
</tr>
<tr>
<td>SKCPHL 05V2626 (H3N2)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>SKCPHL 06V2849 (H3N2)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>SKCPHL 06V2648 (H3N2)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>ODH-99-154 (H3N2)</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Unknown (JH001)</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Texas/36/91 (H1N1)</td>
<td>A</td>
<td>Oseltamivir-Resistant</td>
</tr>
<tr>
<td>Bethesda/5/2006 (H3N2)</td>
<td>A</td>
<td>Amantadine/rimantadine cross-resist</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>NAI cross-resistant in NI assay</td>
</tr>
<tr>
<td>Memphis/20/96</td>
<td>B</td>
<td>NAI cross-resistant in NI assay, NAI</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>sensitive in tissue culture</td>
</tr>
</tbody>
</table>

Drugs. Oseltamivir phosphate was provided by Roche (Hoffmann-La Roche, Switzerland) as a lyophilized powder and was dissolved in sterile distilled water at a concentration of 10mM. Aliquots of the stock solution were kept at -20°C and diluted in cell culture media right before use. Amantadine hydrochloride, bafilomycin A1, and ribavirin were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in water (amantadine) or DMSO (bafilomycin and ribavirin) at a concentration of 5mg/ml, 1mM and 100mM respectively. Nucleoside reverse transcriptase inhibitor zidovudine (AZT) was obtained from NHI AIDS Research and Reference Reagent Program. Compounds QMV-13, QMV-13A, QMV-13B, QMV-15, QMV-15A, QMV-15B, and QMV-15C,
were purchased from ChemBridge Corporation (San Diego, California) as lyophilized powder that was dissolved in DMSO at a concentration of 10mM and stored at -20°C.

**Antibodies.** The following primary antibodies were used: mouse anti-influenza monoclonal antibody blend specific for the nucleoprotein antigen of all subtypes of influenza A (H1N1, H2N2, H3N2 and H5N1) from Chemicon International (Millipore, Billenco, MA); 14c2 (anti-M2, from Dr. Andrew Pekosz, Johns Hopkins University, Baltimore, MD), anti-M1 (HB-64; American Type Culture Collection, ATCC, Manassas, VA) and anti-β-actin from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase-labeled anti-mouse (IgG) secondary antibody was obtained from Sigma-Aldrich (St. Louis, MO).

**Cell protection assay (MTT Assay).** After treatment with compounds and incubation of the cells for 48 hours, colorimetric MTT (tetrazolium) assays (182) were performed in order to measure cell survival. For this, supernatant was removed from each well and 100ul of 0.4mg/ml methylthiazolyldiphenyl-tetrazolium bromide solution (MTT, Sigma-Aldrich, St. Louis, MO) were added followed by 4 hours incubation at 37°C, 5% CO₂. After this, the solution was removed (without disturbing the crystals) and 100ul of DMSO were added per well. After 10 minutes at room temperature the plates were read at 570nm in a microplate reader (Wallac 1420 Victor³V, Perkin Elmer, Waltham, MA).
**Drug susceptibility assays.** Four different methods were used to examine the anti-influenza activity of the compounds:

(i)  **Cell protection assay (MTT).** In this method serial dilutions of the compounds were added 2h before the addition of the virus. Infection of the cells was done with influenza A or B virus at a MOI of 0.005 IU/cell for 48h. After this, supernatants were removed and colorimetric MTT (tetrazolium) assays were done as described above.

(ii) **Measurement of luciferase activity in ELVIRA® FluA cells.** $5 \times 10^4$ Cells/well were plated into white opaque-walled 96-well plates (BD Biosciences, Bedford, MA) pre-treated with 40 $\mu$g/ml human natural fibronectin (BD Biosciences, Bedford, MA). Twenty-four hours later, complete medium was replaced by RMO3T right before the addition of compounds. Cells were infected with influenza A virus at a MOI of 0.05 IU/cell after a 2h pre-incubation with the drugs, followed by the determination of luciferase activity 24h post-infection. This was done by removing the supernatants from the wells, followed by lysis of the cell monolayers (1X Cell culture lysis reagent, Promega Corporation, Madison, WI) and addition of 100ul/well of Luciferase Reagent (Promega Corporation, Madison, WI) after 10 minutes of shaking. Readings of the light intensity (RLU/sec) were done with a microplate reader (Wallac 1420 Victor³ V, Perkin Elmer, Waltham, MA).

(iii) **Viral plaque assay.** Confluent MDCK cells monolayers ($2.5 \times 10^4$ cells/well plated 24h before the experiment) in 96-well plates were washed with PBS
and replenished with 50ul of DMEM containing penicillin-streptomycin (100 U/ml). Serial dilutions of the compounds were added and incubated for 2h before infection with a MOI of 0.005 IU/cell of influenza virus. After 1h incubation, 100ul of 1.2% Avicel overlay media were added per well. This media contained equal amounts of 2.4% Avicel RC/CL (FMC Biopolymer, Philadelphia, PA), a colloidal form of water insoluble cellulose microparticles blended with sodium carboxymethylcellulose, and DMEM containing penicillin-streptomycin (100 U/ml) and 2 µg/ml of TPCK trypsin. 24h after infection, cells were fixed with 50ul of 4% paraformaldehyde solution in PBS at 4°C for 30 minutes. The fixative solution was removed and cells were permeabilized by incubating for 20 minutes with 50ul of 0.5% Triton-X-100 and 20mM glycine in PBS. Immuno-staining was done by incubating cells for 1h with monoclonal antibody specific for the nucleoprotein antigen of influenza virus diluted 1:10,000 with 10% normal horse serum and 0.05% Tween-80 in PBS. The diluted antibody was removed, followed by three washes of 3min each with 0.05% Tween-80 in PBS. Secondary antibody (1:10,000) was incubated for 1h and after the same rounds of washes cells were incubated for 30min with TrueBlue (KPL, Inc., Gaithersburg, MD) a precipitate-forming peroxidase substrate. Distilled water was used to stop the reaction and plates were air-dried and scanned for the records. Plaques were counted manually under the microscope and percentage of infectivity (%
Infectivity) was calculated relative to the number of plaques in the “no drug” wells.

(iv) **Inhibition of virus induced cytopathic effect (CPE)** was determined by visual examination of the cell monolayers (using inverted microscope).

Independently of the method used, antiviral activity of the compounds was calculated as the 50% inhibitory concentration (IC$_{50}$) using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).

**Antiviral activity against other RNA viruses.** Drug susceptibility assays were performed to assess potential antiviral activity of the compounds against WNV, YFV, and hPIV3, as well as HIV-1. For WNV, YFV, and hPIV3, monolayers of Vero (WNV and YFV) and HeLa (hPIV3) cells in 96-well plates (obtained from DIAGNOSTIC HYBRIDS; Athens, OH) were refed with DMEM containing 2% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml). Cells were pre-incubated for 2h at 37°C, 5% CO$_2$ with serial dilutions of the compounds, followed by infection at a MOI of 0.01 IU/cell of each virus. Monitoring of CPE was done daily until maximum cytopathic effect (100% CPE) was observed in the no drug controls. After this, supernatants were removed and colorimetric MTT (tetrazolium) assays were done as described above.

Evaluation of drug susceptibility of HIV-1 was performed using luciferase-tagged reporter viruses obtained from Dr. Jan Weber (Diagnostic Hybrids, Cleveland, Ohio). These replication-competent viruses were created in an intact viral genetic backbone (HIV-1$_{NL4-3}$) by insertion of the firefly luciferase gene between env and nef HIV-1 genes.
without compromising the integrity of the viral genome (276, 277). Construction of both X4- and R5-tropic viruses with the same genotypic backbone involved successful cloning of the HIV-1_YU2 (R5 virus) env gene into HIV-1_NL4-3 (X4 virus). Propagation of the env-recombinant viruses NL4-3-fluc2 and NL4-3-YU2env-fluc2 was done in U87.CD4.CXCR4 and U87.CD4.CCR5 cells, respectively. The same cell lines were used for determination of tissue culture infectivity dose (TCID₅₀) using the end point method of Reed and Muench (209). Quantitation of cells infected with NL4-3-fluc2 and NL4-3-YU2env-fluc2 in the presence of the lead compounds was done using monolayers of the same cell lines specified above plated a day before the experiment into white opaque-walled 96-well plates (BD Biosciences, Bedford, MA) with complete medium (DMEM with 15% FBS, 100 U/ml penicillin-streptomycin, puromycin and geneticin). Serial dilutions of QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B, as well as a single concentration (1 µM) of zidovudine (AZT) as positive inhibition control, were prepared in serum free DMEM and incubated for 2h at 37°C, 5% CO₂ with the cells. Multiplicity of infection (MOI) of 0.01 IU/cell was used to infect the cell monolayers, followed by the determination of luciferase activity 5 days post-infection. For this reporter assay, 100ul of Bright-Glo™ reagent (Bright-Glo™ Luciferase Assay System, Promega Corporation, Madison, WI) were added per well and about two minutes later, light intensity (RLU/sec) was measured with a microplate reader (Wallac 1420 Victor³V, Perkin Elmer, Waltham, MA).
**Drug cytotoxicity.** Cytotoxicity of the compounds was evaluated using the same conditions as for each type of drug susceptibility assay used. Briefly, after incubation of cell monolayers in 96-well plates with increasing concentrations of compounds, the supernatants were removed and cellular toxicity was assayed by three different assays:

(i) *MTT assay*, the colorimetric determination described above which allows for the measurement of cell survival and proliferation when the tetrazolium ring of the substrate is cleaved by active mitochondria, resulting in the formation of blue crystals (182).

(ii) *Trypan blue exclusion*, a method using a vital dye that does not interact with the cell unless the membrane is damaged (76). For this type of assay, previously treated cells were first detached with 20ul of trypsin and neutralized with 60ul of complete DMEM. Trypan blue solution (Mediatech, Inc., Manassas, VA) was added (20ul) and incubated for 5 min at room temperature. After this, the mixture was loaded in a hemocytometer to count the number of viable (unstained) to dead (stained) cells as previously described (76).

(iii) *CellTiter-Glo®* luminescent cell viability assay (Promega Corporation, Madison, WI), a method based on the quantitation of the ATP present as sign of metabolically active cells. This type of assay was performed following manufacturer’s protocol. Briefly, after 48h incubation with the compounds in white opaque-walled 96-well plates (BD Biosciences, Bedford, MA) a volume of CellTiter-Glo® reagent equal to the volume of culture per well was added.
After 10 minutes, a microplate reader (Wallac 1420 Victor3V, Perkin Elmer, Waltham, MA) was used to measure the luminescent signal, which was proportional to the amount of ATP present. For all three methods, the concentration of compound with 50% cytotoxic effect (CC50) was calculated relative to “no drug” control wells.

**In vitro Toxicology Assays (performed at Dyagnostic Hybrids; Athens, Ohio).**

Hepatocytes were isolated from male Sprague Dawley rats that were fed ad libitum and had an average weight of 250-300g. After the effect of intraperitoneal injection of thiopental (anesthetic), liver was perfused with HEPES buffer containing EGTA (0.6mM) followed by a collagenase (100U/ml)/CaCl2 (5mM) solution. Hepatocytes were dissociated mechanically and filtered to obtain a cell suspension. After washing with cold Hanks’ balanced salt solution (HBSS), cell viability was determined by trypan blue exclusion (described above) in order to assure at least 90% viability (if less than 80%, cells were centrifuged on a density gradient). Approximately 40,000 cells per well were plated into collagen treated 96-well plates using growth medium and incubated for about 16h at 37°C, 5% CO2. After this, growth medium was removed and replenished with 100ul of serum free metabolic incubation medium (Williams medium containing 8 µg/ml bovine insulin, 2 µM hydrocortisone, 400 µM GlutaMAX-l (Invitrogen, Carlsbad, California) and 100 U/ml penicillin-streptomycin) (Diagnostic Hybrids; Athens, OH). Serial dilutions of the compounds were added in triplicate and incubated at 37°C, 5% CO2 for 2h. Supernatants were removed and cells rinsed with 1X PBS, after which 100ul
of 0.4 \( \mu \text{g/ml} \) MTT solution were added for the performance of cell survival assays as described elsewhere in this chapter. Concentrations of the compounds at which they become 50% cytotoxic to rat hepatocytes were calculated relative to no drug controls using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).

**Viral Protein Expression.** Confluent MDCK cell monolayers in 6-well plates were pre-treated for 2h with fixed concentrations of the compounds (20 \( \mu \text{M} \), 120 \( \mu \text{g/ml} \) of amantadine) at 37\( ^\circ \text{C} \), 5% \( \text{CO}_2 \), and challenged with influenza A/WSN/33 virus (MOI = 1.5 IU/cell). After adsorption for 1h at room temperature with rocking, cells were washed twice with PBS and replenished with drug-containing DMEM medium with penicillin-streptomycin (100 U/ml) and 2 \( \mu \text{g/ml} \) of TPCK-treated trypsin. After 1h, 3h, 6h, 9h, and 12h incubations, supernatants were harvested and frozen (-80\( ^\circ \text{C} \)) for further TCID\( _{50} \) determinations (method described above). Cell monolayers were lysed with 0.5ml of 1% SDS for the assessment of viral M1 and M2 protein expression by Western Blot as described below.

**Viral Growth Kinetics.** Antiviral activity of the compounds after multiple rounds of replication was evaluated by infecting MDCK cells at a low MOI. These experiments were started in 6-well plates of MDCK cells by pre-incubating for 2h with the compounds (20 \( \mu \text{M} \) or amantadine (120 \( \mu \text{g/ml} \)). Monolayers were infected at a virus MOI of 0.001IU/cell, incubated at room temperature for 1h and washed twice with PBS. DMEM medium with penicillin-streptomycin (100 U/ml) and 2 \( \mu \text{g/ml} \) of TPCK-treated
trypsin containing the same concentration of compounds was added and incubated at 37°C, 5% CO₂, for 1h, 12h, 24h, 36h, or 48h. Supernatants were harvested and frozen (-80°C) for further TCID₅₀ determinations as described above.

**Infectious virus yield (IVY) reduction assay / TCID₅₀ Determination.** Serial 10-fold dilutions of the samples (clarified supernatants or virus to be titrated) were made using DMEM containing penicillin-streptomycin (100 U/ml) and 2 µg/ml of TPCK trypsin, and added to three wells each in 96-well plates of fresh MDCK cells. Five days after infection (incubated at 37°C, 5% CO₂) cell monolayers were fixed with the addition of 100µl of PBS containing 2% paraformaldehyde. After 24h incubation at room temperature, supernatants were discarded and cell monolayers were stained with Giemsa stain solution, which was rinsed-off with tap water after 4 hours at room temperature. Infected wells were defined as those were no staining occurred due to complete cell death. Determination of the tissue culture infectivity dose (TCID₅₀) was done using the end point method of Reed and Muench (209) and expressed as log₁₀ 50% tissue culture infectious doses per milliliter (TCID₅₀) of sample assayed.

**Time-of-drug-addition assays.** Confluent monolayers of MDCK cells in 24-well plates were washed with PBS, replenished with DMEM medium containing penicillin-streptomycin (100 U/ml) and 2 µg/ml of TPCK-treated trypsin (Worthington Biochemical Corp., Lakewood, NJ) and infected with influenza virus (A/WSN/33) at a MOI of 1.5 IU/cell. After adsorption for 1h at room temperature cells were washed three
times with PBS and incubated with the same medium (no FBS) followed by the addition of the compounds (20 µM) or amantadine (120 µg/ml) at different time points, i.e. -2h (2h pre-infection), 0h, 1h, 3h, 6h, and 9h post-infection (wells corresponding to -2h and 0h were replenished with diluted compounds following post-adsorption washes). At 12h after infection, supernatants were harvested, frozen (-80°C), and subjected to TCID$_{50}$ determinations as described above. Remaining cell monolayers were lysed with 1% SDS for the assessment of viral protein expression by Western Blot (method described below).

**Neuraminidase (NA) enzyme inhibition assay.** Initial titrations of NA activity of the virus was performed in order to determine the appropriate working dilution of the particular strain of influenza (93). Briefly, serial dilutions of the virus, were mixed with 75 µM of fluorogenic substrate 2-(4-methylumbelliferyl)D-N-acetylneuraminic acid (MUNANA; Sigma, St. Louis, MO) and incubated at 37°C for 1h. Reactions were stopped by adding 150ul of 0.1M glycine buffer (pH 10.7) containing 25% ethanol. The right working dilution of each particular virus (i.e. 800 to 1200 fluorescence units) was selected after reading the fluorescence of the samples using a microplate reader (Wallac 1420 Victor$^3$V, Perkin Elmer, Waltham, MA) with an excitation wavelength of 365nm and an emission wavelength of 460nm. At this point, evaluation of the ability of compounds to inhibit NA activity was performed as previously described by Gubareva et al. (92). Briefly, equal volumes of the virus and compound were mixed and incubated for 30 min at 37°C, followed by the addition of substrate (MUNANA; Sigma, St. Louis,
MO) at a final concentration of 75 µM. After 1h incubation of the mixture at 37°C the reactions were stopped by the addition of 150ul of the solution already described.

**Immunoblot analyses (Western blotting).** Cells were lysed with 1% SDS and passed through a needle for DNA shearing. After mixing at a 1:1 ratio with 2x loading buffer, 14ul of each sample were loaded onto 10% acrylamide gels to separate the proteins by SDS-PAGE. Next, the resolved proteins were transferred to 0.45um polyvinylidene fluoride (PVDF) membranes (Millipore, Billenco, MA) and blocked overnight at 4°C in PBS containing 5% nonfat milk. Membranes were incubated simultaneously with 14c2 (anti-M2), anti-M1 (HB-64; American Type Culture Collection, ATCC, Manassas, VA) and anti-β-actin (Sigma-Aldrich, St. Louis, MO) as loading control. Incubations with primary antibodies were done in PBS (phosphate-buffered saline) containing 0.1% Tween 20 and 5% nonfat milk for 1h at room temperature followed by a series of washes and incubation for 2h with horseradish peroxidase-labeled secondary antibody. The blots were imaged by using chemiluminescence (Western Lighting Chemiluminiscence Reagent Plus, Perkin Elmer LAS Inc., Boston, MA) and exposure to X-ray films.

**Selection of drug-resistant influenza virus by in vitro passages.** MDCK cells were infected with A/WSN/33 (H1N1) at a MOI of 0.05 IU/cell in 48-well plates in the presence of the compounds starting at a concentration ten-fold lower than their IC_{50}. After 1h incubation at 37°C, 5% CO₂ cell monolayers were washed with PBS and replenished with drug-containing DMEM medium with penicillin-streptomycin (100
U/ml), 2 µg/ml of TPCK-treated trypsin and 0.3% of BSA (EMD Biosciences, Gibbstown, New Jersey). Supernatants were harvested when 80-90% cytopathic effect (CPE) was observed relative to that in the “no drug” control. Aliquots were frozen until viral titer of the preceding passage was determined. This was done by infecting IAV-GFP cell monolayers previously plated onto fibronectin-treated 96-well plates (BD Biosciences, Bedford, MA) with serial 10-fold dilutions of the virus in RMO3T medium (Diagnostic Hybrids; Athens, Ohio) and measuring fluorescence intensity 48h post-infection. Virus was serially passaged at a constant MOI in the presence of increasing concentration of compound (2x) until viral stocks with relatively increased resistance to the respective compound was generated. When development of resistance was suspected, an aliquot of the selected passage was used for virus propagation in MDCK cells. Harvested virus was cleared and filtered, followed by TCID₅₀ determinations in MDCK and ELVIRA®FluA cells as previously described. Drug susceptibility assays with the viruses generated by serial passages were done in parallel with wild type virus, as well as virus passaged in the absence of drug. Both cell survival (MTT assay in MDCK cells) and virus replication (luciferase assay in ELVIRA®FluA cells) were quantified for the determination of IC₅₀ values.
Results

3.1 Lead compounds protect MDCK cells from influenza A/WSN/33 virus, decreasing viral replication.

The ability of QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B to inhibit viral cytopathic effect, plaque formation, and replication was evaluated using influenza A/WSN/33 virus in cell cultures together with known anti-influenza drugs (i.e. oseltamivir, amantadine, ribavirin, and bafilomycine) as controls for the different assays (Fig 3.1).

First, we used the same cell protection assay as for the primary screen. Concentrations of the lead compounds required for the survival of 50% MDCK cells after virus inoculation (IC$_{50}$) ranged from 1.44 µM to 6.60 µM (Table 3.2), confirming the original antiviral activity observed during the screening (Chapter I). Results obtained from the simultaneous determination of A/WSN/33 susceptibility to oseltamivir, amantadine, ribavirin, and bafilomycine also showed the accuracy of this method in measuring inhibition of virus cytopathic effect.

Next, we evaluated the ability of single virus particles to replicate in the presence of these compounds by standard plaque assay (Fig. 3.1). For this, MDCK cells pre-treated with serial dilutions of the compounds were infected with influenza A/WSN/33 virus and incubated for 24h under a low viscosity overlay (Fig 3.2). Similar to results obtained with the MTT assay, the lead compounds inhibited influenza A/WSN/33 virus, with IC$_{50}$ values ranging from 1.00 µM to 8.32 µM (Table 3.2).
Figure 3.1 Drug susceptibility assays by different methods. Influenza A/WSN/33 was evaluated for its sensitivity to QMV-13, QMV-15, their analogues (QMV-13B, 15A, and 15B) as well as oseltamivir (control) in 96-well plates using (A) MTT assay, (B) plaque reduction assay, and (C) ELVIRA®Flu cells.
Table 3.2  IC$_{50}$ Values of lead compounds and control drugs by different methods.

Fifty percent inhibitory concentrations (IC$_{50}$) were determined in triplicate and calculated using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>MTT</th>
<th>Plaque Assay</th>
<th>ELVIRA®FluA cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMV-13</td>
<td>6.50</td>
<td>8.32</td>
<td></td>
<td>7.81</td>
</tr>
<tr>
<td>QMV-13B</td>
<td>1.44</td>
<td>7.54</td>
<td></td>
<td>1.15</td>
</tr>
<tr>
<td>QMV-15</td>
<td>6.60</td>
<td>7.86</td>
<td></td>
<td>7.00</td>
</tr>
<tr>
<td>QMV-15A</td>
<td>4.90</td>
<td>1.00</td>
<td></td>
<td>1.18</td>
</tr>
<tr>
<td>QMV-15B</td>
<td>5.50</td>
<td>4.82</td>
<td></td>
<td>5.40</td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>8.35</td>
<td>0.62</td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>Amantadine</td>
<td>299.06</td>
<td>112.28</td>
<td></td>
<td>32.22</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>10.16</td>
<td>54.58</td>
<td></td>
<td>6.39</td>
</tr>
<tr>
<td>Bafilomycin</td>
<td>0.0072</td>
<td>0.0108</td>
<td></td>
<td>0.0187</td>
</tr>
<tr>
<td></td>
<td>(7.20nM)</td>
<td>(10.84nM)</td>
<td></td>
<td>(18.65nM)</td>
</tr>
</tbody>
</table>

Finally, we assessed the effect of the lead compounds on influenza A/WSN/33 virus replication using the reporter cell line ELVIRA®FluA. Transcription and translation of the “naked” RNA present in these cells by the viral polymerase was inhibited in a dose-dependent manner after treatment with the compounds. As shown in Table 3.2, IC$_{50}$ values ranged from 1.15 µM to 7.81 µM. Inhibition of luciferase expression was also quantified after treatment with serial dilutions of the antiviral drugs amantadine, ribavirin, and bafilomycin. Due to its effect only on late stages of the virus life cycle (release of progeny virions), direct quantification of oseltamivir anti-influenza activity is not possible with this system.
Figure 3.2  Viral plaque reduction assay in MDCK cells under a low viscosity overlay. Sensitivity of influenza A/WSN/33 to serial dilutions of oseltamivir was tested in 96-well plate (concentrations of the drug are indicated below the figure). Plaques were counted manually right after immunostaining, and the resulting numbers were used for the calculation of the IC$_{50}$ value of the drug with the help of a computer software (Prism version 5.00 for Windows).
3.2 Selected compounds are non-cytotoxic at inhibitory concentrations

For validation of the preliminary results, and to search for potential toxicity that could mislead conclusions about their antiviral activity, we evaluated the effect of the lead compounds on all the cell lines used in each method of this characterization process (i.e. MDCK, ELVIRA®FluA, Vero, HeLa, U87.CD4.CCR5, and U87.CD4.CXCR4 cells).

None of the compounds exhibited cytotoxicity in these cell lines as determined by cell survival (MTT) and viability (Trypan Blue exclusion) methods at concentrations of up to 50 µM (Table 3.3). For the purpose stated earlier, incubation of the compounds with these cells corresponded to the times specified in each characterization protocol. Interestingly, higher concentrations (>50 µM) of compounds QMV-15 and its analog QMV-15A seemed to induce changes in the morphology of the cells, with the formation of vesicle-like shapes in their cytoplasm (as observed using the 20X magnification under a regular inverted microscope). Nevertheless, cell metabolism and membrane structure seemed not to be affected as determined by MTT assays and Trypan Blue exclusion, respectively.

To further assess the safety of these compounds in vitro, we quantified cell survival by MTT assay over a period of 24h on the commercially available cell lines: A549 (human lung carcinoma), MRC-5 (human embryonic lung fibroblasts), NCI-H292 (human pulmonary muco-epidermoid carcinoma), WI-38 (human embryonic lung fibroblasts), and NHFL (neonatal human lung). No apparent cytotoxicity was observed
following incubation with the lead compounds at concentrations less than 50 µM (Fig 3.3).

**Table 3.3 CC<sub>50</sub> determinations of selected compounds in cell lines used for their characterization.** Incubation periods with the drugs were 48h for MDCK, 24h for ELVIRA®Flu cells, and 5 days for Vero, HeLa, U87.CD4.CCR5, and U87.CD4.CXCR4 cells, according to protocols followed for IC<sub>50</sub> determinations with those cell lines. (a) Cell proliferation assays (MTT). Percentages of cell survival were calculated relative to “no drug” controls. (b) Trypan blue exclusion to evaluate effect of compounds and control drugs on cell viability. (c) Cell survival determined by quantitation of the ATP present as sign of metabolically active cells (CellTiter-Glo®, Promega). CC<sub>50</sub> values were calculated from triplicate determinations using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).
<table>
<thead>
<tr>
<th>Compound (μM)</th>
<th>MDCK cells</th>
<th>ELVIRA®FluA cells</th>
<th>Vero cells</th>
<th>HeLa cells</th>
<th>U87 CD4 CCX4</th>
<th>U87 CD4 CCR5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Trypan Blue&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MTT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Trypan Blue&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MTT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MTT&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>QMV-13</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>QMV-13B</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;20</td>
</tr>
<tr>
<td>QMV-15</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>QMV-15A</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50</td>
<td>&gt;20</td>
<td>10</td>
</tr>
<tr>
<td>QMV-15B</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Aramadine</td>
<td>&gt;1650</td>
<td>&gt;1650</td>
<td>&gt;1650</td>
<td>&gt;1650</td>
<td>&gt;1650</td>
<td>&gt;1650</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>1023.7</td>
<td>1023.7</td>
<td>1023.7</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bafilomycin</td>
<td>0.1</td>
<td>&gt;0.1</td>
<td>0.1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Figure 3.3 CC$_{50}$ values of selected compounds in different immortalized human epithelial cell lines mimicking the respiratory tract. Cell survival assays (MTT) were performed in triplicate after 24h incubation with the compounds of the cell lines: (A) A549, (B) MRC-5, (C) NCI-H292, (D) WI-38, and (E) NHFL. Figure (F) summarizes the CC$_{50}$ determinations of amantadine in all five cell lines. Percentages of cell survival were calculated relative to “no drug” controls. CC$_{50}$ values were determined with the help of Prism version 5.00 for Windows (GraphPad Software, San Diego, California).
3.3  

*In vitro* Toxicology

Because of their resemblance in metabolic patterns to human hepatocytes, short-term cultures of primary rat hepatocytes were used as the *in vitro* model for drug toxicity studies. Mitochondrial activity of these cells was quantified as the non-liver specific toxicity marker (216). For this, isolation of Sprague Dawley rat hepatocytes was done according to procedures developed by Diagnostic Hybrids (Athens, Ohio), followed by MTT assays 2h after incubation of the cells with QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B, as well as oseltamivir and amantadine. As shown in Figure 3.4, none of the lead compounds was toxic to rat hepatocytes at their known inhibitory concentrations. Moreover, concentrations as high as 50 µM did not reach levels lower than 50% cell survival.
Figure 3.4 In vitro toxicology of lead compounds. Cell survival assays were performed in Sprague Dawley Rat hepatocytes after incubation with increasing concentrations of the selected compounds. 50% Cytotoxic Concentrations (CC\textsubscript{50}) that resulted higher than 50μM (>50μM) for all the compounds were calculated using a computer software (Prism version 5.00 for Windows) starting from the relative absorbances (570nm) obtained after MTT assays. Cellular toxicity of oseltamivir and amantadine were tested in parallel as control for the experiments.
3.4 Replication of influenza A viruses is inhibited by QMV compounds

Antiviral activity of QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B was evaluated against different strains of influenza A (H1N1) and influenza A (H3N2) in ELVIRA®FluA cells (Table 3.4). Of the influenza A (H1N1) viruses, the clinical isolate C1d was the most susceptible to all five compounds with IC$_{50}$ values from 0.03 µM to 2.71 µM. Influenza virus strains A/WS/33, A/Texas/36/91 (wild type), and A/Texas/36/91 (oseltamivir resistant, His274Tyr) were sensitive to inhibition in the 0.41 µM to 11.84 µM range. Activities of the lead compounds against the laboratory adapted A/PR/8/34 virus were lower, at 5.59 µM to 18.13 µM. More important, all QMV compounds showed antiviral activity similar to that observed with amantadine in these H1N1 viruses.

Having demonstrated their susceptibility to the QMV compounds, strains of influenza A/Texas/36/91 (wild type), and A/Texas/36/91 (oseltamivir resistant) were further evaluated for the confirmation of their phenotypic differences. Because the same reporter cell system could not be used to directly test for NA inhibition, we resorted to cell protection assays (MTT) in MDCK cells (Fig 3.5). Indeed, A/Texas/36/91 (oseltamivir resistant) did not show any susceptibility to oseltamivir even at 50 µM concentration, with the wild type strain having an IC$_{50}$ value of 7.17 µM.

Finally, replication of eight laboratory adapted and clinical isolates influenza A (H3N2) viruses as well as an unsubtyped strain, were inhibited by all five compounds using the ELVIRA®FluA cells system (Table 3.4). Overall, the clinical isolates showed higher sensitivity to the compounds (IC$_{50}$ 0.80 to 9.78 µM).
Table 3.4 IC$_{50}$ values of lead compounds against different strains of influenza A virus. Drug susceptibility determinations were performed in triplicate using ELVIRA®Flu cells. Relative Luminescence was measured 24h after infecting with an MOI of 0.05 IU/cell and percentage of infection was calculated relative to “virus only” controls. Fifty percent inhibitory concentrations (IC$_{50}$) were calculated using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amantadine (µM)</th>
<th>QMV-13 (µM)</th>
<th>QMV-13B (µM)</th>
<th>QMV-15 (µM)</th>
<th>QMV-15A (µM)</th>
<th>QMV-15B (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/H1N1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/WS/33</td>
<td>26.60</td>
<td>7.44</td>
<td>10.92</td>
<td>3.48</td>
<td>4.08</td>
<td>10.16</td>
</tr>
<tr>
<td>C1d</td>
<td>22.50</td>
<td>0.03</td>
<td>0.07</td>
<td>0.04</td>
<td>2.71</td>
<td>0.03</td>
</tr>
<tr>
<td>A/Texas/36/91 (Parent)</td>
<td>103.41</td>
<td>10.84</td>
<td>5.85</td>
<td>0.41</td>
<td>4.4</td>
<td>6.44</td>
</tr>
<tr>
<td>A/Texas/36/91 (Mutant: Oseltamivir resistant)</td>
<td>82.44</td>
<td>11.84</td>
<td>7.51</td>
<td>5.47</td>
<td>3.66</td>
<td>5.01</td>
</tr>
<tr>
<td>A/H3N2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Udorn/72</td>
<td>46.84</td>
<td>10.41</td>
<td>15.02</td>
<td>5.19</td>
<td>9.53</td>
<td>10.95</td>
</tr>
<tr>
<td>A/Bethesda/5/2006 (AMT resist, NAI resist in NA assay)</td>
<td>207.69</td>
<td>1.08</td>
<td>0.21</td>
<td>0.74</td>
<td>0.18</td>
<td>0.86</td>
</tr>
<tr>
<td>A/HongKong/8/68</td>
<td>202.20</td>
<td>10.61</td>
<td>16.31</td>
<td>6.45</td>
<td>5.86</td>
<td>16.23</td>
</tr>
<tr>
<td>SKCPHL 06V2648</td>
<td>34.86</td>
<td>0.83</td>
<td>0.94</td>
<td>6.66</td>
<td>4.37</td>
<td>6.86</td>
</tr>
<tr>
<td>SKCPHL 06V2849</td>
<td>223.76</td>
<td>3.46</td>
<td>0.99</td>
<td>5.05</td>
<td>3.8</td>
<td>3.14</td>
</tr>
<tr>
<td>SKCPHL 05V2626</td>
<td>40.69</td>
<td>1.69</td>
<td>1.06</td>
<td>5.24</td>
<td>9.78</td>
<td>6.53</td>
</tr>
<tr>
<td>ODH-99-154</td>
<td>42.28</td>
<td>1.05</td>
<td>0.93</td>
<td>4.44</td>
<td>2.79</td>
<td>5.06</td>
</tr>
<tr>
<td>SKCPHL 05V2625</td>
<td>18.53</td>
<td>0.8</td>
<td>1</td>
<td>2.75</td>
<td>5.07</td>
<td>5.68</td>
</tr>
<tr>
<td>A/unsubtyped</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Victoria/3/75</td>
<td>142.65</td>
<td>0.7</td>
<td>0.99</td>
<td>1.38</td>
<td>4.07</td>
<td>7.2</td>
</tr>
</tbody>
</table>
Figure 3.5 Wild-type A/Texas/36/91 vs. oseltamivir-resistant A/Texas/36/91.

Susceptibility of the wild-type and mutant strain to the NA inhibitor oseltamivir was tested in triplicate by cell protection assay (MTT) in MDCK cells. Percentages of cell survival at different concentrations of the drug were calculated relative to “no virus” controls.
3.5 QMV compounds also inhibit Influenza B virus replication

Anti-influenza B activity was evaluated *in vitro* by cell protection assays in MDCK cells. Compounds QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B, demonstrated potent efficacy against a panel of laboratory strains and recent clinical isolates of influenza B virus as shown in Table 3.5. All four strains were susceptible to the compounds with IC$_{50}$ values ranging from 0.08 µM to 8.95 µM. Potency of the NA inhibitor oseltamivir against these influenza B virus strains was comparable to that of the lead compounds (IC$_{50}$ ranging from 0.68 to 7.22 µM). In contrast, amantadine proved to be ineffective against these viral strains (IC$_{50}$ from 99.7 to 140.30 µM) (Table 3.5).
Table 3.5 IC\textsubscript{50} values of lead compounds against different strains of influenza B virus. Cell protection assays (MTT) were performed in triplicate 5 days after infecting with an MOI of 0.005 IU/cell and absorbances (570nm) were used to calculate the percentages of cell survival relative to “no virus” controls. Fifty percent inhibitory concentrations (IC\textsubscript{50}) were calculated using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amantadine ((\mu M))</th>
<th>QMV-13 ((\mu M))</th>
<th>QMV-13B ((\mu M))</th>
<th>QMV-15 ((\mu M))</th>
<th>QMV-15A ((\mu M))</th>
<th>QMV-15B ((\mu M))</th>
<th>Oseltamivir ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL/1739/54</td>
<td>692.07</td>
<td>0.37</td>
<td>0.13</td>
<td>0.49</td>
<td>0.25</td>
<td>7.82</td>
<td>0.68</td>
</tr>
<tr>
<td>Memphis/20/96 (Mutant: NAI resist in NI assay)</td>
<td>928.27</td>
<td>0.08</td>
<td>0.12</td>
<td>0.14</td>
<td>0.44</td>
<td>0.85</td>
<td>2.93</td>
</tr>
<tr>
<td>Maryland/1/59</td>
<td>895.20</td>
<td>0.46</td>
<td>0.28</td>
<td>1.25</td>
<td>0.36</td>
<td>1.3</td>
<td>7.22</td>
</tr>
<tr>
<td>JH001 R&amp;D DHI</td>
<td>659.65</td>
<td>0.47</td>
<td>0.34</td>
<td>7.77</td>
<td>1.37</td>
<td>8.95</td>
<td>4.7</td>
</tr>
</tbody>
</table>
3.6 **Antiviral Spectrum of lead compounds**

To evaluate the specificity of the lead compounds (i.e., ability to inhibit the replication of other RNA viruses), cell protection assays were performed using WNV, YFV, and hPIV3 viruses. Vero (WNV, YFV) and HeLa (hPIV3) cells were infected with 0.01 MOI of each virus after a 2h pre-incubation with serial dilutions of the compounds, followed by quantification of cell survival 48h p.i. by MTT assays. Interestingly, cytopathic effects caused by these three RNA viruses were not inhibited by any of the lead compounds even at the highest concentrations tested (50 µM) (Fig 3.6).

Using a different approach, we evaluated the antiviral activity of all five compounds against HIV-1. For this, X4- and R5-tropic HIV-1 luciferase-tagged env-recombinant viruses (NL4-3-fluc2 and NL4-3-YU2env-fluc2, respectively) were used to infect cells pre-treated with serial dilutions of the compounds (Fig 3.7). Effectiveness of this system for drug susceptibility determinations of HIV-1 was confirmed with the complete inhibition of luciferase activity by 1 µM of AZT 5 days post-infection (data not shown). When tested, compounds QMV-13, QMV-13B, and QMV-15B did not display any antiretroviral activity (Fig 3.7). However, luciferase expression was inhibited in a dose-dependent manner by compounds QMV-15 and QMV-15A, inhibiting both X4 and R5 viruses with similar potency (Table 3.6).
Figure 3.6 Antiviral activity against different RNA viruses. Cell proliferation assays (MTT) were used to determine the percentages of cell survival in the presence of the lead compounds after infection with (A) yellow fever virus (YFV), (B) west Nile virus (WNV), (C) human parainfluenza virus type 3 (HPIV-3) at MOI of 0.01 IU/cell. As described earlier, percentages of cell survival were calculated relative to “no virus” controls.
Figure 3.7 Evaluation of antiviral activity against Human Immunodeficiency Virus Type 1 (HIV-1). The properties of the lead compounds were tested against two envelope-recombinant viruses, (A) CXCR4-tropic HIV and (B) CCR5-tropic HIV, both bearing firefly luciferase as a reporter gene. Five days after infection in the presence of various concentrations of the drugs, relative luminescence was measured and percentage of infection was calculated relative to “virus only” controls.
Table 3.6 Antiretroviral activity of compounds QMV-15 and QMV-15A. Fifty percent inhibitory concentrations (IC₅₀) are the mean of triplicate determinations and were calculated using Prism version 5.00 for Windows (GraphPad Software, San Diego, California).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>U87.CD4.CCR5</th>
<th>U87.CD4.CCX4</th>
</tr>
</thead>
<tbody>
<tr>
<td>QMV-15</td>
<td>2.48</td>
<td>1.90</td>
</tr>
<tr>
<td>QMV-15A</td>
<td>4.58</td>
<td>2.27</td>
</tr>
</tbody>
</table>

3.7 Effect of delay of treatment initiation on anti-influenza activity

Time-of-drug-addition experiments were performed as a first attempt to further identify the inhibitory effect of the lead compounds on different stages of viral infection (Fig 3.8). As shown in Figure 3.9, viral yields determined after a single round of influenza A/WSN/33 virus replication showed clear trends of inhibition by compounds QMV-13, QMV-15, and their analogs. Clear suppression of virus infection was observed when 20 µM concentrations of QMV-13 and QMV-13B were added between -2h and 3h post-infection, with a gradual decrease in their inhibitory effect when treatment was delayed for 6h or longer. Pre-treatment of cells for 2h and the addition of compounds QMV-15 and analogs at the time of infection resulted in almost a threefold decrease of infectious virus titers. Yield reduction assays were also performed from supernatants taken after time-of-drug-addition experiments with amantadine and oseltamivir (Fig 3.9). The results obtained verified the mode of action of both drugs, validating the usefulness of these experiments for the characterization of the lead compounds.
Figure 3.8 Design of Time-of-Drug-Addition experiments. Single cycle virus growth conditions were used in order to estimate the step of the viral life cycle that is inhibited by the lead compounds. MDCK monolayers were infected at a MOI of 1.5IU/cell. Adsorption was allowed for 1h followed by serial washes with PBS and replenishing with fresh medium. Compounds were added to the cells at fixed concentrations (20uM) at different time points during the incubation. 12h after infection supernatants and cells were harvested for determination of infectious titers (TCID$_{50}$) and viral protein expression, respectively.
**Figure 3.9 Delay of treatment initiation on antiviral activity.** Infectious titers of A/WSN/33 (H1N1) virus harvested from time-of-drug-addition experiments were calculated by the end point method of Reed and Muench (209), and the titers were expressed as $\log_{10}$ 50% tissue culture infectious dose per milliliter of medium assayed ($\log_{10}\text{TCID}_{50}$). (The red line indicates the $\log_{10}\text{TCID}_{50}$ of “no drug” controls).
In addition to virus yield determinations, expressions of M1 and M2 viral proteins were quantified from cell monolayers following time-of-drug-addition assays (Fig 3.10). After immunoblotting and acquisition, images were processed and analyzed with a software (QuantiScan version 2.1, Biosoft; Cambridge, United Kingdom) that allowed the quantification of proteins expression and their normalization relative to a common loading control (β-Actin). Results showed clear trends in the inhibition of M1 and M2 levels with all five compounds when 20 µM were added to the cells early during infection. Moreover, comparable to the data obtained from infectious virus titers, a strong inhibition of M1 and M2 protein expression after treatment with QMV-15 and QMV-15A suggested potent antiviral activity when added at the time of infection or earlier (Fig 3.10).
Figure 3.10 M1 and M2 viral protein expression after different times of treatment initiation. Cell monolayers from time-of-drug-addition experiments were harvested after 12h incubation and analyzed by Western Blot. Levels of expression of M1 and M2 proteins after treatment with (A) amantadine, (B) oseltamivir, (C) QMV-13, (D) QMV-13B, (E) QMV-15, (F) QMV-15A, and (G) QMV-15B were quantified using QuantiScan version 2.1, and calculated relative to β-Actin (loading control).
E

F

G
3.8 Lead compounds inhibit viral protein synthesis in infected MDCK cells

MDCK cell monolayers were lysed at different time points during high MOI influenza A/WSN/33 infection (1.5 IU/cell) (Fig 3.11). Homogenized lysates were loaded onto acrylamide gels followed by immunoblotting procedures. After Western blotting for M1 and M2 virus protein expression we found that, after exposure to 20 µM of the compounds levels of these two proteins were significantly reduced (Fig 3.12). Amounts of M1 and M2 in the no drug controls, which were detectable starting from 9h post-infection, were quantified and used as 100% values for calculations of protein expression after normalization against β-Actin (loading control). As a result, we described compounds QMV-15 and QMV-15A as the most potent in inhibiting M1- and M2-expression even after 12h p.i., followed by QMV-13B, QMV-13, and QMV-15B which caused a delay in the expression of these two proteins. Also, drug controls amantadine and oseltamivir were included in these experiments to validate the results and to serve as references for the description of potential mechanisms of action of the QMVs. As expected, amantadine showed an early reduction in protein expression whereas oseltamivir did not show any effect on cell-associated virus.
Figure 3.11 Design of Viral Protein Expression Experiments. The antiviral effect of the lead compounds was evaluated at various steps of the virus life cycle. After 2h pre-incubation with the compounds at fixed concentrations, a MOI of 1.5IU/cell of A/WSN/33 (H1N1) virus was used to infect MDCK cell monolayers. Adsorption was allowed for 1h after which the inoculum was removed and cells were washed. At 1h, 3h, 6h, 9h and 12h p.i. supernatants and cells were harvested for determination of infectious titers (TCID_{50}) and viral protein expression, respectively.
Figure 3.12 Viral protein expression at different steps of the virus life cycle. Infected cell monolayers were harvested at different time points after infection with A/WSN/33 (H1N1) at an MOI of 1.5IU/cell in the presence of (A) amantadine, (B) oseltamivir, (C) QMV-13, (D) QMV-13B, (E) QMV-15, (F) QMV-15A, and (G) QMV-15B. Western Blot analyses were performed and levels of expression of M1 and M2 proteins were quantified using QuantiScan version 2.1 and calculated relative to β-Actin (loading control).
Furthermore, virus growth curves were performed on MDCK cells with supernatants harvested from the same experiments in order to additionally assess the effect of the described conditions on extracellular virus yield (Fig 3.13). Virus titers determined from cells pre-treated with QMV-15, QMV-15A, and QMV-15B showed similar growth kinetics and indicated a sustained decrease of up to two folds in the amount of infectious virus produced starting at 9h p.i. when compared to untreated controls. Conversely, the inhibitory effect of compounds QMV-13 and QMV-13B on virus replication started as early as 6h p.i. with one-fold reduction compared to untreated samples. This difference remained constant until 12h p.i. for viruses in the presence of QMV-13, while those treated with QMV-13B reached maximum growth at that time point (12h p.i.).
Figure 3.13 Virus yield reduction after high MOI infection in the presence of the lead compounds. The concentration of infectious virus present in the supernatant after a single round (MOI 1.5IU/cell) of replication of A/WSN/33 (H1N1) in the presence of fixed concentrations of the compounds was determined at different steps of viral infection. Virus titers were calculated by the end point method of Reed and Muench (209), and expressed as log$_{10}$ 50% tissue culture infectious dose per milliliter of medium assayed (log$_{10}$TCID$_{50}$).
3.9 Influenza A/WSN/33 virus growth is inhibited by QMV compounds

Influenza virus A/WSN/33 (H1N1) was exposed to 20 μM of the lead compounds during multiple rounds of virus replication (0.001 IU/cell for 48h), during which supernatants were harvested at different time points to measure their virucidal effect on extracellular virus by infectious virus yield-reduction assays (Fig 3.14). Inhibitory effects of compounds QMV-15, QMV-15A and QMV-13B were observed as two- to five-fold reductions in virus titers almost throughout the whole incubation period (48h p.i.) (Fig 3.15), kinetics that were comparable to those observed in the presence of amantadine and oseltamivir. Compared to no drug controls, compounds QMV-13 and QMV-15B suppressed virus reproduction about two folds at 12h p.i. (approximately one round of replication), after which extracellular virus titers reached maximum levels (Fig 3.15).
Figure 3.14 Design of Virus Yield Reduction assays after low MOI infection. The anti-influenza effect of the lead compounds after multiple rounds of replication was evaluated at various steps of the virus life cycle. After 2h pre-incubation with the compounds at fixed concentrations, a MOI of 0.001 IU/cell of A/WSN/33 (H1N1) virus was used to infect MDCK cell monolayers. Adsorption was allowed for 1h after which the inoculum was removed and cells were washed. At 1h, 12h, 24h, 36h and 48h p.i. supernatants and cells were harvested for determination of infectious titers (TCID<sub>50</sub>).
Figure 3.15 Virus yield reduction following multiple rounds of replication in the presence of the lead compounds. Concentrations of infectious virus present in the supernatants after multiple rounds of replication of A/WSN/33 (H1N1) (MOI 0.001IU/cell) in the presence of fixed concentrations of the compounds was determined at different steps of viral infection. Virus titers were calculated by the end point method of Reed and Muench (209), and expressed as $\log_{10} 50\%$ tissue culture infectious doses per milliliter of medium assayed ($\log_{10} \text{TCID}_{50}$).
3.10 Effect of lead compounds on the neuraminidase activity of influenza A and B viruses

The potential effect of the lead compounds on the neuraminidase activity (NA) of influenza type A subtypes N1 and N2 was tested using a fluorometric assay (93). Dilutions of strains A/WSN/33 (H1N1) and A/Udorn/72 (H3N2) containing between 1,200 and 2,000 fluorescent units were selected for the performance of these NA susceptibility assays. Following incubation with the compounds, relative fluorescent units (RFUs) were measured and quantification of NA activity was done relative to no drug controls. Concentrations of the positive control oseltamivir required for reducing NA activity by 50% (IC$_{50}$) were 153.80 nM and 78.66 nM for A/WSN/33 (H1N1) and A/Udorn/72 (H3N2), respectively. However, none of the lead compounds showed any inhibitory effect against these viruses NA (Fig 3.16). In addition, all five compounds were tested against B/Memphis/20/96, an oseltamivir-resistant influenza B virus strain carrying the Arg152Lys mutation in its NA gene. As shown in Figure 3.16, neither the lead compounds, nor the drug control oseltamivir were capable of inhibiting the activity of this virus enzyme.
Figure 3.16 Effect of lead compounds on the NA activity of influenza virus. The effect of the lead compounds as well as oseltamivir on the NA activity of influenza virus strains: (A) A/WSN/33 (H1N1) \((IC_{50} \text{ Oseltamivir} = 153.8 \text{nM})\) (B) A/Udorn/72 (H3N2) \((IC_{50} \text{ Oseltamivir} = 78.66 \text{nM})\), and (C) B/Memphis/20/96 (Mutant: NAI resist in NI assay), were evaluated using a fluorometric assay.
3.11 *In vitro selection of drug resistant influenza A variants*

Serial passages of influenza A/WSN/33 virus were done in MDCK cell cultures in the presence of increasing concentrations of the lead compounds. As controls for this selection procedure, wild type virus was passaged in the absence of drug, as well as in the presence of the well known anti-influenza drugs amantadine and oseltamivir. As shown in Figure 3.17, concentrations were increased up to 48 µM for compounds QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B; 250 µg/ml for amantadine, and 12 µM for oseltamivir. Starting from 10 times less their IC₅₀, concentrations were doubled with every passage when the titers were high enough to keep a constant MOI (0.05 IU/cell). In cases when this was not possible (too low TCID₅₀), the preceding passage was repeated in the presence of either the same or lower concentration of drug in order to exert less selective pressure and allow for more virus replication to take place. The latter approach explains the drops in concentrations observed in Figure 3.17, especially during passages with QMV-13, QMV-15B and oseltamivir.
**Figure 3.17** Selection of drug-resistant influenza virus. In vitro passage of influenza A/WSN/33 virus in the presence of increasing concentrations of the lead compounds was done in MDCK cells for the development of drug-resistant variants. Indicated by the red rectangles are the passages propagated in culture and further tested for phenotypic changes. Concentration of the compounds at those times were 6-, 11-, 5-, 10-, 9-, and 3.5-folds higher than the IC$_{50}$ values of QMV-13, QMV-13B, QMV-15, QMV-15A, QMV-15B, and amantadine (AMT), respectively.
After reaching at least five times the IC$_{50}$ values of each drug (Fig 3.17), aliquots of the passaged viruses were propagated in MDCK cells followed by TCID$_{50}$ determinations in two different cell lines (MDCK and ELVIRA®FluA). Drug susceptibilities of these viruses, including the one passaged in the absence of drug as well as wild-type, were evaluated by virus replication assays (luciferase assay in ELVIRA®FluA cells). As shown in Figure 3.18, there was no significant difference in the IC$_{50}$ values of the QMV-resistant viruses compared to the control viruses. On the other hand, influenza A/WSN/33 passaged in the presence of amantadine showed to be phenotypically different to the corresponding wild type virus with 24-fold shifts in IC$_{50}$ values (Fig 3.18) as determined by luciferase assay.
Figure 3.18 Drug susceptibility determinations of serially passaged influenza viruses. Potential drug-resistant variants, in parallel with wild-type influenza A/WSN/33 as well as A/WSN/33 passaged in the absence of drug were evaluated for their sensitivity to each lead compound: A) QMV-13, B) QMV-13B, C) QMV-15, D) QMV-15A, E) QMV-15B, and F) amantadine. Fifty percent inhibitory concentrations (IC$_{50}$) were determined in triplicate in ELVIRA®FluA cells using Prism version 5.00 for Windows (GraphPad Software, San Diego, California)
Discussion

The immediate threat of a new influenza pandemic has prompted the obvious need for novel antiviral drugs that could be used in the prophylaxis and treatment of this highly pathogenic disease. With the contribution to that need as our goal, we have identified five small molecules that displayed promising anti-influenza properties. The main objective of this chapter was to further describe the \textit{in vitro} antiviral activity of these lead compounds.

We started the process of characterization of compounds QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B by testing their influenza virus-inhibitory effect using different methods. Cell protection assays, virus plaque reduction assays, and influenza A-detecting luciferase assays (ELVIRA®FluA) resulted in low IC$_{50}$ values during drug susceptibility assays with influenza A/WSN/33 virus, thus confirming their sustained antiviral effect. Moreover, the use of the ELVIRA®FluA system not only helped us to accurately measure the inhibition of virus replication by the lead compounds, but also provided us with an indirect indication of their possible mechanism of action. Interestingly, the fact that luciferase expression as indicative of infection in this virus-detection system is proportional to viral polymerase recognition and replication of the RNA present in the reporter cells (167), strongly suggested that post-transcriptional stages of the virus life cycle could not be considered as potential targets of these compounds.
Effect of the lead compounds on cell integrity was further evaluated in vitro by MTT assays performed in cultures of various immortalized cell lines that closely mimic the human tissue targeted by influenza virus in vivo. CC$_{50}$ values significantly higher than the inhibitory concentrations (>50 µM) of these compounds in A549 human lung carcinoma cells, MRC-5 human embryonic lung fibroblasts, NCI-H292 human pulmonary muco-epidermoid carcinoma, WI-38 human embryonic lung fibroblasts, and NHFL neonatal human lung cells suggest that treatment in vivo with the lead compounds would not be harmful to the human airway epithelium. It is evident that additional studies will be needed in order to move forward the potential development of these compounds as actual anti-influenza drugs. In addition, knowing the inherent link between toxicity and metabolism, we focused on the liver as one of the main organs involved in the biotransformation of drugs (216). Short-term cultures of rat hepatocytes previously reported to be useful in hepatotoxicity, genotoxicity, and biokinetic studies (21), were used as our liver-based in vitro model for drug toxicity studies. Results from these assays indicated that none of our lead compounds have detrimental effects on rat hepatocytes at the concentrations tested (up to 50 µM), suggesting their in vivo safety thus defining them as qualified candidates for the potential development of novel anti-influenza treatments.

The ability of various influenza A virus strains to replicate in vitro was inhibited in the presence of the lead compounds at IC$_{50}$ values ranging from 0.03 to 11.84 µM for H1N1 subtypes, and 0.18 to 15.02 µM for H3N2 strains. Differences in these inhibitory concentrations may be attributable to the previously described variability of infectiousness in cell culture within different strains of influenza virus (232). Influenza B
viruses were also successfully inhibited by all five compounds, as determined by cell protection assays. The low IC$_{50}$ values obtained with all the B strains tested (between 0.12 and 8.95 µM) eliminated the possibility of an influenza A-specific mechanism of action, and suggested a common target for the compounds in both influenza A and B virus infection.

A potential broad antiviral activity of these compounds was further evaluated using other RNA viruses such as WNV, YFV, hPIV3, and HIV-1. Contrary to the absence of an inhibitory effect against WNV, YFV, and hPIV3 by the lead compounds, significant antiviral activity was obtained against HIV-1 with QMV-15 and one of its analogs. Interestingly, antiretroviral activity of QMV-15 has been previously identified by Jegede et.al. after screening the same library of small molecules (Small Molecule Screening Core of the Cleveland Clinic; Cleveland, Ohio) for novel anti-HIV-1 compounds (134). This study aimed particularly to the identification of HIV-1 coreceptor antagonists with the use of replicative-competent fluorescent viruses capable of distinguishing between CCR5- and CXCR4-specific drugs (134, 277). Unpublished results from this screening describe the identification of QMV-15 as a potent HIV-1 inhibitor (IC$_{50}$ values 0.15 µM and 4.25 µM in MT4 cells and PBMC), and characterization of its mechanism of action is underway (134). Interestingly, the two QMV-15- and QMV-15A-sensitive RNA viruses, influenza (segmented negative-sense genome) and HIV-1 (single stranded positive-sense genome), share the unique property of transcribing and replicating in the nucleus, contrary to most RNA viruses such as WNV (positive-strand RNA flavivirus), YFV (positive-strand RNA flavivirus), and
hPIV3 (non-segmented negative strand RNA virus) which replicate in the cytoplasm (75, 144). These results not only confirmed the specific antiviral activity of the lead compounds, but also indicated a slightly broader spectrum of action for QMV-15 and QMV-15A.

A very useful tool in the characterization of the antiviral activity of QMV-13, QMV-15, and their analogs (QMV-13B, QMV-15A, and QMV-15B) was the analysis of time-of-drug addition experiments. Because the period of time when compounds are most active is related to the step of the virus life cycle targeted, we have studied the effect of delay of treatment initiation on the anti-influenza activity of the lead compounds. Various models of influenza virus replication have estimated the completion of a single round of infection to take approximately 5h, with nuclear transport of vRNPs taking place only 30 min post-infection, and the switch from vmRNA production to virus genome replication 3h after virus attachment and internalization (which occur 2-5 min, and 15-20 min post-infection respectively) (Fig 3.19) (7, 231). Addition of oseltamivir and amantadine at different time points throughout influenza A/WSN/33 virus infection clearly reflected their patterns of inhibition. Sustained reduction of virus titers after the addition of oseltamivir up to 6h after infection confirmed inhibition of late stages of virus replication, known in this case to be the release of progeny virions (49, 139). Also, time-of-amantadine-addition experiments described the pattern of inhibition of virus uncoating, with high titers detected only when adding this drug 3h p.i. (Fig 3.19). Reductions of virus yields were observed when QMV-13 and QMV-13B were added on or after 3h after infection, suggesting early stages of the virus reproductive cycle (possibly from virus
attachment up to replication) as their potential targets. Leads QMV-15 and QMV-15A, seem to share a common mechanism of action by exerting their antiviral activity only when added before or at the time of infection. This narrower timeframe between virus attachment and vmRNA synthesis (before 3h p.i.), together with the earlier assumption that the activity of these compounds might be related to the common property of influenza and HIV-1 of replicating in the nucleus has led us to speculate about nuclear transport as the possible target of QMV-15 and QMV-15A. On the other hand, QMV-15B did not show a marked inhibition profile in these experiments, although its action appears to be similar to that of QMV-15 and QMV-15A. Results from these experiments using virus yield determinations were supported by similar inhibition trends observed in expressions of M1 and M2 proteins from cell lysates. Altogether, time-of-drug addition experiments characterized our lead compounds as early inhibitors of influenza virus infection (Fig 3.19). In agreement with the latter, preliminary data obtained from biochemistry assays with QMV-13 and QMV-15 performed by Dr. Paul C. Roberts (Virginia Tech College of Veterinary Medicine; Blacksburg, Virginia), have suggested that the early inhibitory activity of QMV-15 may be due to its targeting of entry, uncoating, or nuclear transport. Also, these preliminary results indicated that QMV-13 has an effect on the expression of late virus proteins, which could be due to inhibition of virus replication (Fig 3.19).

Influenza virus gene expression is divided in two phases according to quantitative changes of transcription of individual RNA segments occurred during replication (152). The early phase comprises the coupled synthesis of vRNA and vmRNA, while during the
second one vmRNA is shut down (152, 225). Independently from the latter, protein synthesis of influenza virus occurs throughout infection reaching sustained maximal rates during the second phase, which indicates that protein synthesis is largely determined by the rate of vmRNA synthesized during the early phase of viral gene expression (225). With this in mind, we evaluated the effect of the lead compounds on virus-specific protein expression in order to better understand their mechanisms of action. Viral proteins M1 and M2 were the focus of our studies due to their relevant differences. The matrix protein (M1), the most abundant protein in the virion, is encoded by the unspliced M-specific vmRNA (219). This early protein is believed to be structural in function and required for nuclear-cytoplasmic transport of the RNPs as well as for virus maturation (70, 219). The ion channel protein M2 on the other hand, is encoded by the spliced M-specific vmRNA, and it is therefore synthesized later in the replication process (219). Expression of M1 and M2 proteins was determined after single rounds of influenza A/WSN/33 virus infection in the presence and absence of the lead compounds. Since separate antibodies were used for the detection of each protein, comparison between M1 and M2 levels of expression would not be accurate. However, simultaneous immunoblotting and quantification allowed us to reliably analyze the effect of the lead compounds on both proteins under the exact same conditions. In line with our previous results, all five compounds caused a time-dependent inhibition of M1 and M2, with compounds QMV-15 and QMV-15A displaying the most potent and prolonged effects. As expected, virus-specific protein expression was not reduced in the presence of our NA inhibitor (oseltamivir) due to the highly detectable levels of initial infection generated by
the high MOI used in these experiments. These results, coupled with the reduced extracellular virus yields from the same set of experiments, provided more evidence that QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B target early stages of infection (Fig 3.19).

Influenza A/WSN/33 growth curves were also deduced from virus yield reduction assays after low MOI (0.001 IU/cell) infections in the presence of 20 µM of the lead compounds. Results from these experiments described the ability of QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B to inhibit influenza virus after multiple rounds of replication, suggesting that their antiviral properties not only affect those virions responsible for initial infection but also virus progeny.

An even more specific approach was taken with the performance of neuraminidase (NA)-inhibition assays. Influenza virus NA, in a balanced interrelationship with hemagglutinin (HA), allows for the successful spread of infection by removing the HA binding receptors from cell and virion surfaces during progeny release (136). Relevance of NA in influenza virus replication led us to the assessment for a potential effect of the lead compounds on this enzyme even though the data obtained from time-of-drug-addition experiments, protein expression determinations, as well as luciferase assays using the reporter cell line ELVIRA®FluA consistently characterized these compounds as early inhibitors of influenza infection. Results obtained from fluorometric NA-inhibition assays with influenza A/H1N1, A/H3N2, and B isolates showed that the leads do not exert a direct inhibitory effect on NA at any of the concentration tested, eliminating this enzyme as their target. Oseltamivir on the other
hand, effectively inhibited the sensitive virus strains tested resulting in IC$_{50}$ values of 153.80 nM and 78.66 nM, differences that can be explained by the previously reported variability in NA activities among influenza virus strains (136). Moreover, the confirmed lack of inhibitory activity of QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B on the sialidase activity of an influenza B variant with the Arg152Lys mutation completely disqualified these compounds as possible NA-inhibitors.

Development of virus resistance in vitro has been widely used to depict the molecular targets of drugs by identifying mutations conferring resistance to each particular compound (63, 115, 206). Identification and characterization of these drug resistant variants is the final evidence of the molecular target for any given compound. Therefore, we intended to select for influenza virus variants with reduced susceptibility to QMV-13, QMV-13B, QMV-15, QMV-15A, or QMV-15B. Serial passages of influenza A/WSN/33 virus in MDCK cell cultures were done in the presence of increasing concentrations of the lead compounds as described in previous studies (89, 179, 292). After 12 to 20 passages with concentrations four- to eleven-fold higher than their calculated IC$_{50}$ values (16 µM to 48 µM) no phenotypic changes were observed in these viruses. However, it is also possible that the fitness of this virus may require prolonged passage in further-increasing concentrations of the lead compounds for the emergence of resistant variants. Moreover, difficulty in the selection of an oseltamivir-resistant A/WSN/33 variant as an internal control for our experiments has added to our hypothesis that extra rounds of passages would be necessary for the emergence of drug-specific mutations. Yet, it is important to take into consideration that after continuous passages
we have reached concentrations of the lead compounds that started to cause toxic effects on the cells (>50 μM), which has forced us to maintain fixed concentrations for various passages with increases of only 0.2-folds at the time. Therefore, we could assume that under not so stringent conditions of selective pressure, development of QMV-resistant variants could be considerably delayed or even impeded. Based on the lack of resistance emergence observed so far, we could infer that the lead compounds may not be selectively targeting the virus; conversely, they could be affecting the host cell or a mechanism related to it.

Although the exact mechanism by which compounds QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B exert their anti-influenza activity will still be subject of further investigation, we have been able to repeatedly characterize them as early inhibitors of viral infection, finding that has significantly reduced the number of possible targets and, even more, may increase their therapeutic value as novel therapeutics due to their potential applicability as prophylactic agents (Fig 3.19). Description of the susceptibility of a cohort of influenza A and B virus strains to these compounds, as well as their safety in a variety of cell cultures modeling human tissue, have also provided us with a better understanding of the extent in the effectiveness of these promising anti-influenza drug candidates.
Figure 3.19 Inhibition of the influenza virus reproductive cycle by antiviral drugs.

Represented in this diagram are the dynamics of the virus life cycle based on in vitro simulations done by Sidorenko et al. (231). According to this model, virion attachment occurs 2-5 min p.i., followed by internalization in approximately 10 min. After 15-20 min virions inside the cell start to uncoat and release their vRNPs which reach the nucleus in 30 min p.i. Adamantane derivatives and compounds BL-1743 and Bafilomycin A1 block this uncoating step. According to our study, the steps described until now could be targeted by QMV-15, QMV-15A, and QMV-15B (inhibit replication before 3h p.i.). Compounds QMV-13 and QMV-13B could be targeting up to virus replication (inhibit infection after up to 3h). Transcription of vmRNA starts immediately in the nucleus, and is switched to replication 3h p.i. This processes can be blocked by inhibitors of the virus transcriptase, (i.e. flutimide, L735,882, 2-FDG, T-705), antisense oligonucleotides and IMPDH-inhibitors (i.e. ribavirin, T-CONH2, LY217896). Virus assembly is followed by the release of new progeny 5h p.i., which can be blocked by the NA inhibitors oseltamivir, zanamivir, and those under development (i.e. peramivir, A-315675, Siastatin B).
CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Summary

With the present study we have joined researchers around the world in the common goal of identifying new molecules for the future development of potent anti-influenza drugs that will be effective against all virus strains without causing side effects. It is the imminent threat of another influenza pandemic, together with the high numbers of deaths due to annual epidemics, what constantly remind us about the lack of resources currently available and therefore the need for an effective approach to fight this emerging disease.

Our contribution to this search started with the identification of two compounds (QMV-13 and QMV-15) with promising anti-influenza properties from a library of 34,000 organic small molecules (Chapter II). Selection took into account not only the antiviral effectiveness of the primary hits, but also their in vitro innocuity. The novel structural scaffolds of these two compounds were carefully analyzed and taken as references for the identification of analogs, which properties in cell culture partially uncovered the role of some of QMV-13 and QMV-15 substituents and provided insights about possible improvements using combinatorial chemistry. Due to their sustained antiviral properties, three of these analogs were included in the group of “leads”.
Following up on these promising findings was the characterization of the lead compounds described in the third chapter of this study (Chapter III) where QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B were objects of meticulous analyses. Inhibition of a potent laboratory adapted influenza A virus strain (A/WSN/33) by different methods, in addition to antiviral determinations against a panel of laboratory adapted, clinical isolates, as well as drug-resistant strains of influenza A and B viruses confirmed the broad spectrum of anti-influenza activity of the lead compounds. In the same line with the latter results, specificity of the compounds was determined when in vitro replication of WNV, YFV, and HPIV3 was not inhibited. Similar results were obtained with HIV-1, although its infectivity was inhibited only by QMV-15 and QMV-15A. Equally important, in vitro safety of all five compounds was described in continuous cell lines mimicking human epithelium targeted by influenza virus infection, followed by toxicology assays in rat hepatocytes.

Time-of-drug-addition experiments and protein expression determinations together with virus growth kinetics after single and multiple rounds of replication suggest that early stages of virus replication may be the target of these compounds. This observation was further supported by the inability of the leads to interfere with the sialidase activity of neuraminidase late in the influenza virus life cycle. A more detailed identification of the molecular targets of QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B is underway with the prolonged passage of the virus under the selective pressure of these compounds. We expect that specific selected mutations (amino acid
substitutions likely responsible for their drug resistance) will reveal those gene(s) targeted by these molecules.

The results from this study represent the first steps into the extensive process of drug discovery which is known to take up to 10 years altogether (66). Lead optimization (medicinal and combinatorial chemistry) and development (pharmacology, pharmacokinetics, toxicology, among other studies) result in confirmed drug candidates from which only 5 out of 5000 are considered safe for testing in humans, trials that may take from 3 to 6 years (66). Therefore, the design of novel approaches for the identification of molecules with antiviral properties is of utmost importance, same as the need for increased numbers of leads to widen the options in this search. The identification of QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B together with the characterization of their anti-influenza A and B activities, not only signify the accomplishment of our initial goals but also our first contribution to the urgent need for novel anti-influenza treatments.

**Future Directions**

**4.1 Cross-resistance patterns of QMV-resistant variants**

*In vitro* selection for drug resistant variants is a common tool used for the identification of the molecular target of compounds under research (115). Moreover, the same approach provides with information about potential mutations likely to emerge during therapy, probable rates of emergence of resistant variants, as well as useful predictions of possible cross-resistance in the clinical setting (179).
As described in Chapter III, we have started the process of influenza A/WSN/33 resistance selection to QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B. *In vitro* selection of drug resistant viruses may take a few weeks or several months (up to more than 32 months) depending on the virus used and the potency of the anti-viral agent. In our case, although no significant phenotypic changes have been identified yet in these viruses, serial passages in further-increasing concentrations of the lead compounds are still underway. Should QMV-resistant variants emerge, identification of specific mutations will be identified by nucleotide sequencing. Furthermore, we would create recombinant viruses harboring these mutations using reverse genetics and (i) recombination of the targeted gene into the influenza A/WSN/33 backbone and/or (ii) the insertion of the specific mutation in the known wild type strain of the virus by site directed mutagenesis. Independently of the approach used, we would test the susceptibility of the constructed viruses against the respective compound to confirm the gene as the antiviral target.

On the other hand, emergence of resistance to currently approved anti-influenza drugs has been documented during treatment (39). Although less common than resistance to M2 inhibitors which are known exhibit complete cross-resistance between them (74, 103), resistance to NA inhibitors is being reported more often (39). Cross-resistance between oseltamivir and zanamivir has been observed in clinical isolates (84), and what is more, influenza variants resistant to both oseltamivir and amantadine/rimantadine have been documented (88, 213) Therefore, not only it is of great urgency to identify novel compounds active against influenza virus, but also to develop those active against drug-
resistant strains. With this in mind, drug susceptibility determinations with QMV-13, QMV-13B, QMV-15, QMV-15A, QMV-15B, amantadine, and oseltamivir, will be performed against each of the QMV-resistant variants for the identification of possible cross-resistance to these inhibitors.

4.2 Assessment of compound-target interactions

Usually, genetic approaches like the ones proposed above are not sufficient for a detailed characterization of the mechanism of action of a compound. Once the mutations identified by those means are described as the potential target, studies of the interaction between the compound and that residue(s) need to be done to confirm that the resistance observed is indeed due to alterations in that specific interaction (145). This is usually accomplished with the performance of biochemical assays that will depend on the nature of the target and the system under study. Given the fact that we do not have a confirmation of potential targets to our lead compounds, it is difficult to predict the assays that would best suit our purposes. However, based on our present findings, experiments evaluating initial steps of influenza infection could be proposed in advance, such as:

- Hemadsorption assays for the examination of virus binding, which consist in the infection of cell monolayers with influenza virus and examination for their binding to erythrocytes using an optical microscope (148). Less binding of cells pre-treated with the lead compounds and challenged with the virus would point at HA (hemagglutinin) as their target.
• Lipid mixing, hemifusion/fusion assays that would provide us with information about a potential effect of compounds QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B on influenza virus fusion. A protocol developed by Wessels et.al. (279) using fluorescent influenza virus particles to track and measure their fusion to supported lipid bilayers would be useful for this purpose.

• Hemolysis assays as an alternative to the experiment described above, would allow monitoring of virus fusion by measuring hemolysis of erythrocytes caused by fusion of membrane fragments containing HA with the erythrocytes (125).

• In vitro vRNA synthesis studies would reveal any interference by the lead compounds in this specific process. As described by Momose et.al. (180), this type of in vitro RNA synthesis can be done by pre-treating vRNP with nucleases and providing the required reagents and conditions (i.e. RNA template of negative polarity, RNP cores, dNTPs, etc.) for the extension of vRNA models of certain length in nucleotides.

• Keeping in mind the possibility that our lead compounds might be targeting host cells rather than the virus directly, we could study the interferon (IFN) responses, crucial components of host antiviral defense, as potential targets of our lead compounds. For this, immunoassay detection (enzyme-linked immunosorbent assays) of IFN after influenza infection of cell monolayers in the presence or absence of the compounds
could be a straightforward approach; same as determination of mRNA expression of these cytokines by Real-Time PCR (247). Also, a possible downstream effect of the compounds could be evaluated by using a tetracycline-regulated 3T3 L1 cell line that inducibly expresses wild-type PKR (9). As one of the best characterized cellular genes induced by IFN, the double-stranded RNA (dsRNA)-depended, serine/threonine protein kinase (PKR) is a good indicative of IFN signaling, and thus any alteration in the already described path to Fas expression and apoptosis after influenza infection in this cell line would corroborate an effect of the lead compounds (10).

4.3 Drug combinations against influenza virus

Combination therapy is a known approach proven to be advantageous during treatment of viral infections. Combination of drugs targeting different steps of the virus life cycle not only can drastically improve inhibition of replication as a result of additive or synergistic effects, but also offers the possibility of combating virus resistance (145). The latter is explained by the fact that the probability of a virus developing resistance to multiple different drugs is very low, which is also true in the case of infection with already resistant variants. Moreover, the usually undesired emergence of resistant mutations to one of the drugs could render a less fit virus that may be more sensitive to the other drugs involved in the treatment (64, 145). Therefore, availability of multiple antiviral drugs acting on different virus targets is necessary for the design of successful
combination regimens. In order to assess the usefulness of our lead compounds for this particular purpose, we proposed the evaluation of potential additive, synergistic, or even antagonistic effects when used in combination against influenza A/WSN/33 *in vitro*. Cell protection assays (MTT) were performed in triplicate on MDCK cells pre-treated with eight two-fold dilutions below and above the IC$_{50}$ values of the lead compounds combined with amantadine or oseltamivir. Percentages of cell survival that fell within an intermediate range of protection (>$0\%$ and $<100\%$) were used to calculate the combination index (CI) with CompuSyn version 1.0.1 software (Chou and Martin, 2005) for each pair of drugs (Table 4.1) (44).

**Table 4.1** Effect of drug combinations on MDCK cells survival after infection with *influenza virus* A/WSN/33. Combination Index (CI) of indicated drug co-treatments determined at 95% inhibitory effect (ED$_{95}$) were calculated using CompuSyn version 1.0.1 software. Combination Indexes are indicative of synergism, additive effect, or antagonism when CI $<$ 1, CI = 1, or CI $>$ 1, respectively (45).

<table>
<thead>
<tr>
<th>CI at ED$_{95}$</th>
<th>Amantadine</th>
<th>Oseltamivir</th>
<th>QMV-13</th>
<th>QMV-13B</th>
<th>QMV-15</th>
<th>QMV-15A</th>
<th>QMV-15B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td></td>
<td>0.10182</td>
<td>0.66659</td>
<td>0.37985</td>
<td>0.30511</td>
<td>0.00163</td>
<td>0.18502</td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>0.10182</td>
<td></td>
<td>0.48463</td>
<td>0.16132</td>
<td>0.28589</td>
<td>0.00331</td>
<td>0.22279</td>
</tr>
</tbody>
</table>

The combination index (CI) of a co-treatment is a quantitative measure of the degree of drug interaction in terms of synergism and antagonism for a given endpoint of the effect measurement, and it follows the diagnostic rule that CI $<$ 1, CI = 1, and CI $>$ 1.
indicate synergism, additive effect, and antagonism, respectively (45). In addition to the CI values, classic isobolograms were generated that provided illustrations of the equipotent combinations of the two drugs involved as well as the resulting combination data points at 50%, 75%, and 90% fractional inhibitions (i.e., Fa, percentage inhibition/100) (Fig 4.1). Diagnosis of the resulting effect out of this type of report is based on the area of the graph where the data points fall, where the lower-left of the hypotenuse represents synergism, while upper-right and on the hypotenuse indicate antagonism and additive effect, respectively. According to the results obtained from these preliminary experiments, QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B show significant synergism in combination with both antiviral drugs (Table 4.1). However, out of the eight different concentrations tested, only two or three fit the software’s parameters (>0% and <100%), which made the results partially virtual (predicted by the software). Therefore, further determinations are needed using a wider range of drug concentrations that would provide us with more useful data. This would most likely result in a higher number of values that may fall within the intermediate range of protection (>0% and <100%) required by the software to give a more reliable statistical report. Moreover, the use of a more sensitive method (e.g. ELVIRA®FluA reporter system) still keeping the constant ratio combination design may be useful in that the same number of concentrations tested would probably result in more suitable values. Even though these preliminary data needs to be confirmed in future experiments, the results obtained seem encouraging and supportive of the potential use of these compounds as novel therapeutic approaches.
Figure 4.1 Isobolograms for drug combination treatments of MDCK cell monolayers. These figures indicate the equipotent combinations of various doses of (A) QMV-13B and amantadine, and (B) QMV-13 and oseltamivir on MDCK cells survival after infection with influenza virus A/WSN/33. Isobolograms were generated using CompuSyn version 1.0.1 software.
A

B
4.4 Antiviral activity against avian influenza A/H5N1 virus

Currently, the highly pathogenic avian influenza H5N1 virus represents an immediate pandemic threat due to its repeatedly proven ability to infect humans (avian-to-human infection), and its already documented human-to-human transmission after prolonged contact with infected patients (266). Hence, it is not difficult to foresee the possibility of this virus to undergo direct adaptation or reassortment with an existing influenza strain after human or pig co-infection (199). Moreover, likelihood of any of the two phenomena to occur increases with factors such as the ongoing globalization and easiness of travel, as well as the ever-increasing world population (199). Should the H5N1 virus become a pandemic, we will not have enough resources to control it unless new prophylactic and therapeutic treatments become available. It has been reported that currently approved M2 inhibitors do not inhibit the H5N1 subtype (160), and only the NA inhibitors have shown to be active against it (157), although resistance to oseltamivir has already emerged during treatment due to incomplete viral suppression (61).

As a response to this threat, we have started a collaboration with Dr. Ruben Donis from the Molecular Virology and Vaccines branch at the Influenza Division of the CDC (Atlanta, GA) to conduct a series of experiments for the in vitro evaluation of QMV-13, QMV-13B, QMV-15, QMV-15A, and QMV-15B against the highly pathogenic avian influenza A/H5N1 virus. For this, preliminary experiments will be performed infecting pre-treated MDCK cell monolayers as described in this study, followed by in vitro drug susceptibility assays in differentiated primary human tracheobronchial epithelial cells (HTBE) as previously done by this group (42). These experiments will allow us to
identify a potential effect of the lead compounds on avian H5N1 virus replication, results that ideally would be further tested against possible reassortants of this virus with human influenza strains (H5N1 and H3N2) that have been already generated using reverse genetics by Donis et.al. (42).

4.5 Future High Throughput Screenings for Antiviral Compounds

In Chapter I we have shown that use of MTT assays for in vitro high throughput screenings can efficiently identify compounds inhibiting any of the influenza viral targets, thus protecting the cells from infection. Moreover, this HTS system proved useful in the elimination of compounds displaying inherent toxicity, avoiding high numbers of false positives otherwise common after primary screenings with other systems. Future application of this cell-based system in HTS for anti-influenza compounds could incorporate variations that would adapt it to different parameters depending on the expectations. Such modifications could include (i) increasing the incubation period with the virus, which would identify only the most potent compounds; (ii) increasing or decreasing the multiplicity of infection (MOI) to adjust the sensitivity of the assay, or (iii) adding the compounds at different times after virus inoculation, which would narrow the search to compounds targeting from post-entry to post-translational steps in the virus life cycle. Moreover, this HTS system could be adapted to be used in the identification of compounds against other viruses producing lytic infection in cell culture such as West Nile and Yellow Fever viruses.
An alternative and innovative assay with great potential for use in future high throughput screenings is the influenza-A virus detecting luciferase reporter cell line ELVIRA®FluA developed by Diagnostic Hybrids Inc. (Athens, Ohio). Although commercialized for diagnosis purposes in medical laboratories, the ELVIRA®FluA cell line constitutes an advantageous resource for big scale HTS due to its proven sensitivity to a wide variety of influenza A virus strains, as well as the short duration of its reporter assay. Anticipating our involvement in new influenza antiviral screenings in the near future, we have validated the protocol used in Chapter III (drug susceptibility determinations) for its use under HTS conditions (Fig 4.2). Briefly, a MOI of 0.05 IU/cell is used to infect ELVIRA®FluA cell monolayers in white opaque-walled 96-well plates (BD Biosciences, Bedford, MA) after a 2h pre-incubation with the compounds, followed by the determination of luciferase activity 24h post-infection. For higher efficiency during HTS, we replaced the Luciferase Reagent (Promega Corporation, Madison, WI) with BrightGlo (Promega Corporation, Madison, WI), which does not require the removal of infectious supernatant for cell lysis and further quantification of light intensity (RLU/sec). Validation of these conditions for HTS was done by testing three 96-well plates containing infected and uninfected controls, resulting in a $Z'$ value of 0.67 (Fig 4.2). This result, as a measurement of assay performance and consistency (290), indicated the reliability of the ELVIRA®FluA cell line as a HTS system. We are in the process of establishing scientific and commercial collaborations with different institutions and pharmaceutical companies to use ELVIRA®FluA, and the still in development
ELVIRA®FluB systems to identify novel compounds and future drugs against the influenza virus.
**Figure 4.2 Z’ Value Determination of ELVIRA®Flu HTS system.** White solid bottom 96-well plates were seeded with ELVIRA®Flu cells (5x10^4/well). The next day cells were washed with PBS and seeded with RMO3T medium. Diluted virus (0.05 MOI) was added to 48 wells of each plate and incubated for 24h, followed by performance of luciferase assays. The Z’ value for this HTS system as the average of three different determinations was 0.660.
## APPENDIX A

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td>3’-azido-2’,2’dideoxythymidine; Zidovudine</td>
</tr>
<tr>
<td>CC	extsubscript{50}</td>
<td>Fifty percent Cytotoxic Concentration</td>
</tr>
<tr>
<td>CCR5</td>
<td>CC-Chemokine Receptor 5</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
</tr>
<tr>
<td>CD8	extsuperscript{+}</td>
<td>CD8-expressing T lymphocytes</td>
</tr>
<tr>
<td>CO	extsubscript{2}</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic Effect</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary Ribonucleic acid</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytolytic T Lymphocytes</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC-chemokine receptor 4</td>
</tr>
<tr>
<td>DHHS</td>
<td>U.S. Department of Health and Human Services</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GTP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human Immunodeficiency Virus type 1</td>
</tr>
<tr>
<td>hPIV3</td>
<td>human Para-Influenza Virus 3</td>
</tr>
<tr>
<td>HTS</td>
<td>High Throughput Screening</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Fifty percent inhibitory concentration</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IND</td>
<td>Investigational New Drug</td>
</tr>
<tr>
<td>IU</td>
<td>Infectious Unit</td>
</tr>
<tr>
<td>IVY</td>
<td>Infectious Virus Yield</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NAI</td>
<td>Neuraminidase Inhibitor</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>NEP</td>
<td>Nuclear Export Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NIAID</td>
<td>National Institute Allergy and Infectious Diseases</td>
</tr>
<tr>
<td>NISN</td>
<td>Neuraminidase Inhibitor Susceptibility Network</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS1</td>
<td>Non Structural protein 1</td>
</tr>
<tr>
<td>M</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase A</td>
</tr>
<tr>
<td>PB1</td>
<td>Polymerase B1</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase B1</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Luminescence Units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interference Ribonucleic acid</td>
</tr>
<tr>
<td>SNS</td>
<td>Strategic National Stockpile</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded Ribonucleic acid</td>
</tr>
<tr>
<td>TCID\textsubscript{50}</td>
<td>Fifty percent Tissue Culture Infectivity Dose</td>
</tr>
<tr>
<td>TI</td>
<td>Therapeutic Index</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TPCK</td>
<td>L-1-(tosyl-amido-2-phenyl)ethyl chloromethyl ketone</td>
</tr>
<tr>
<td>vmRNA</td>
<td>viral messenger Ribonucleic acid</td>
</tr>
<tr>
<td>vRNA</td>
<td>viral Ribonucleic acid</td>
</tr>
<tr>
<td>vRNP</td>
<td>viral Ribonucleoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile Virus</td>
</tr>
<tr>
<td>YFV</td>
<td>Yellow Fever Virus</td>
</tr>
</tbody>
</table>
REFERENCES


Ref Type: Electronic Citation

Ref Type: Electronic Citation


35. **CDC.** 2006. High Levels of Adamantane Resistance Among Influenza A (H3N2) Viruses and Interim Guidelines for Use of Antiviral Agents - United States, 2005-2006 Influenza Season, p. 44-46. MMWR.


Ref Type: Electronic Citation

Ref Type: Electronic Citation

Ref Type: Electronic Citation
40. CDC, CCID NCIRD. Health Alert. CDC Recommends against the Use of Amantadine and Rimantadine for the Treatment or Prophylaxis of Influenza in the United States during the 2005-06 Influenza Season. http://www.cdc.gov/flu/han011406.htm. 2008. CDC. Ref Type: Electronic Citation

41. CDC, H. Center for Disease Control and Prevention, U.S. Department of Health and Human Services "Strategic National Stockpile (SNS)". 2005. Ref Type: Electronic Citation


61. de Jong, M. D., T. T. Tran, H. K. Truong, M. H. Vo, G. J. Smith, V. C. Nguyen, V. C. Bach, T. Q. Phan, Q. H. Do, Y. Guan, J. S. Peiris, T. H. Tran,


71. FDA. Center for Drug Evaluation and Research. "FDA Approved drugs and Therapeutic Biological Products". Office of Training and Communications, Division of Information Services.

Ref Type: Electronic Citation


84. GlaxoSmithKline. Relenza. Highlights of Prescribing Information. GlaxoSmithKline. 2008. Ref Type: Electronic Citation


117. HHS. U.S. Department of Health and Human Services, "Pandemic Flu". Ref Type: Electronic Citation

118. HHS. United States Department of Homeland Security. "Flu Pandemic". 2006. Ref Type: Electronic Citation

119. HHS. U. S. Department of Health and Human Services. "HHS Pandemic influenza plan". 2007. Ref Type: Electronic Citation


Ref Type: Electronic Citation


Ref Type: Electronic Citation


Ref Type: Electronic Citation


265. **Ulmanen, I., B. A. Broni, and R. M. Krug.** 1981. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription. Proc Natl Acad Sci U S A **78**:7355-7359.


Ref Type: Electronic Citation


