An Investigation into Novel Drug Candidates Against Paediatric Viral Pathogens

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Abstract

Human viral pathogens are a serious cause of illness worldwide, and are responsible for a significant number of infectious diseases. Recurring respiratory infections account for the greater part of the disease burden associated with viral infections in infants, while we see more and more emerging viruses affecting this population, especially in Asia.

Human respiratory syncytial virus (hRSV) and human parainfluenza type-3 virus (hPIV-3) are the main aetiological agents of acute lower respiratory tract infection in infants worldwide. Analogously, enterovirus 71 (EV71) is a major cause of hand foot and mouth disease (HFMD) in Asia, an ordinarily benign paediatric disease that in some cases develops to a severe neurological infection. Sadly, neither vaccines nor drugs are to date available to prevent or treat infection by either of these viruses.

The work presented in this Thesis is focussed on the discovery of novel inhibitors of hPIV-3, hRSV and EV71 infection, by using multidisciplinary approaches and various drug discovery strategies.

Human parainfluenza type-3 virus possesses a surface haemagglutinin-neuraminidase (HN), responsible for binding to sialic acid-containing host cell receptors, sialic acid cleavage, and activation of viral fusion. It is a key protein in viral replication, and a target of choice for drug discovery. Target-based drug discovery approaches were therefore carried out to identify 2 distinct types of HN inhibitors.

The first inhibitor, a sialic acid-based inhibitor named compound 10, is the result of rational, computer-aided drug design that led to the synthesis of novel sialic acid-based compounds. It was designed based on the crystal structure of hPIV-3 HN in complex...
with zanamivir, and a report describing the possibility of HN to accommodate larger ligands due to the opening of a flexible loop in the vicinity of the protein’s active site. Several derivatives of Neu5Ac2en, an early sialic acid-based neuraminidase inhibitor, with bulky substituents located at the C-4 and C-5 positions on the sialic acid framework were therefore synthesised. They were shown to inhibit the neuraminidase and haemagglutination functions of the HN protein, as well as infection in vitro, as a consequence of locking open a flexible loop through protein engagement of the incorporated functional groups.

The second inhibitor, the approved drug suramin, was identified by enzymatic screening of a drug library on HN sialidase. Virological methods allowed the characterisation of the drug as both an entry and release inhibitor of infection in vitro, while the combined use of enzyme kinetics and competition-saturation transfer difference NMR spectroscopy (STD-NMR) of whole virus particles in the presence of suramin and a competitive inhibitor of HN led to the conclusion that suramin is a non-competitive inhibitor of the sialidase. Furthermore, the drug was shown to act in synergy with competitive inhibitors of the enzyme, such as the anti-influenza drug zanamivir, to block infection in vitro.

The potent hRSV inhibitor, cyclopamine (CPM), was identified by phenotypic screening of a compound library and was described as an inhibitor of late events in hRSV replication. CPM is a known inhibitor of a host cell signalling pathway, which was shown by chemical profiling to have no role in hRSV infection in vitro. With the use of resistance selection, the hRSV anti-terminator of transcription and polymerase co-factor M2-1 was identified as a potential target for the compound. As a consequence, CPM was tested in minigenome experiments that enabled the expression of hRSV replication complex in transfected cells. CPM was shown to potently inhibit M2-1-dependent replication complex, in a dose-dependent manner, while having a deleterious effect on M2-1 protein levels. In addition, CPM efficacy in the mouse model is described.

Finally, the drug suramin was discovered as an inhibitor of EV71 infection by phenotypic screening of a drug library, carried out to select repurposable drugs for HFMD.
The drug was characterized as an entry inhibitor of EV71 infection \textit{in vitro}, and STD-NMR experiments led to the conclusion that suramin binds to the virus capsid via its multiple sulfonated groups. These groups were shown to be critical for suramin anti-EV71 potency. Additionally, the pharmacological profile of the drug was evaluated in monkeys, and suramin demonstrated antiviral efficacy both in the mouse and the rhesus monkey models of infection.

Together, these compounds provide novel molecular scaffolds and give new directions for the future development of potent antivirals.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the Thesis contains no material previously published or written by another person except where due reference is made in the Thesis itself.

_____________

Benjamin Bailly
Acknowledgements

To begin with, I would like to express my most sincere gratitude to my supervisors, Prof. Mark von Itzstein, Dr. Patrice Guillon, and Prof. Ralf Altmeyer, for their invaluable guidance and support through the course of this Ph.D. Mark, thank you for your ever enlightening input at and for always being there, even from the other side of the ocean. Patrice, you have since then become a dear friend and you have taught me a lot, I am extremely grateful. Ralf, thank you for giving me this opportunity to come working with you in Shanghai. This has been a intense and rewarding experience, personally and professionally.

I also wish to deeply thank the Institute for Glycomics, Griffith University, the Institut Pasteur Shanghai and the Institut Pasteur International Network for their technical, academical and financial support, and for providing me with the structure necessary to achieve this candidature. On a more personal note, I ought to acknowledge Fiona Crone and Yimei Zheng for their administrative help, they have done a fantastic job in making this Australia/China collaboration run flawlessly.

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Marine, my everything, nothing would ever be the same without you by my side. You inspire me and animate me, I will be eternally grateful.
Acknowledgement of Papers

Included in this Thesis

Included in this Thesis are papers in Chapters 2, 3 and 4 which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details and the status for these papers including all authors are:

Chapter 2


Chapter 3

Chapter 4


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in each paper.

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Additional Material Arising from this Thesis

The following patent was filed subsequent to the discovery reported in Chapter 3, p. 129:

Title
Inhibitors and Antiviral Drugs Designed to Target the Human Respiratory Syncytial Virus.

Inventors
Altmeyer, R., Bailly, B. & Gang, Z.

Assignee
Institut Pasteur Shanghai – Chinese Academy of Sciences

Filing Date
7th of July, 2014

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<td>3D</td>
<td>3-dimensional</td>
</tr>
<tr>
<td>ALRI</td>
<td>acute lower respiratory tract infections</td>
</tr>
<tr>
<td>BLA</td>
<td>biologic licence application</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>COM</td>
<td>composition of matter</td>
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<td>CPE</td>
<td>cytopathic effect</td>
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<td>CPM</td>
<td>cyclopamine</td>
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<td>CV-A16</td>
<td>Coxsackie virus A16</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>EV71</td>
<td>enterovirus 71</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GE</td>
<td>gene-end region</td>
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<tr>
<td>GS</td>
<td>gene-start region</td>
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<tr>
<td>HAE</td>
<td>human airway epithelial cells</td>
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<td>HFMD</td>
<td>hand, foot and mouth disease</td>
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<td>hMPV</td>
<td>human metapneumovirus</td>
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<tr>
<td>hPIV</td>
<td>human parainfluenza virus</td>
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<td>hRSV</td>
<td>human respiratory syncytial virus</td>
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<td>HTS</td>
<td>high throughput screening</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<td>MAb</td>
<td>monoclonal antibody</td>
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<td>MD</td>
<td>molecular dynamics</td>
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<td>MOU</td>
<td>method of use</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NDA</td>
<td>new drug application</td>
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<td>Newcastle disease virus</td>
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<td>NF-κB</td>
<td>nuclear factor κB</td>
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<td>NME</td>
<td>new molecular entity</td>
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<td>NMR</td>
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<td>NOE</td>
<td>nuclear Overhauser effect</td>
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<tr>
<td>nt</td>
<td>nucleotide</td>
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<td>ORF</td>
<td>open reading frame</td>
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<td>PBD</td>
<td>phenotype-based drug discovery</td>
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<td>PSGL-1</td>
<td>human P-selectin glycoprotein ligand 1</td>
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<td>qPCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SAR</td>
<td>structure activity relationship</td>
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<td>SCARB2</td>
<td>scavenger receptor B2</td>
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<td>Shhp</td>
<td>sonic hedgehog signalling pathway</td>
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<tr>
<td>siRNA</td>
<td>short-interfering RNA</td>
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<tr>
<td>Smo</td>
<td>smoothened protein</td>
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<td>SPR</td>
<td>surface plasmon resonance</td>
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<td>saturation transfer difference nuclear magnetic resonance</td>
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<td>TBD</td>
<td>target-based drug discovery</td>
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<tr>
<td>VLP</td>
<td>virus-like particle</td>
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vRNA  viral ribonucleic acid

**Constants and variables**

CC\textsubscript{50}  concentration of a compound that results in 50% cytotoxicity *in vitro* (M)

[I]  substrate concentration (M)

[S]  inhibitor concentration (M)

$\Delta G_{\text{binding}}$  binding free energy change (kcal/mol)

$\Delta G_{\text{desolv}}$  desolvation free energy change (kcal/mol)

$\Delta G_{\text{elec}}$  electrostatics free energy change (kcal/mol)

$\Delta G_{\text{H-bond}}$  hydrogen-bonding free energy change (kcal/mol)

$\Delta G_{\text{tor}}$  torsion free energy change (kcal/mol)

$\Delta G_{\text{vdW}}$  van der Waals free energy change (kcal/mol)

$\Delta G^\circ$  Gibbs free energy change in standard conditions (J)

$\Delta H^\circ$  enthalpy change in standard conditions (J)

$\Delta S^\circ$  entropy change in standard conditions (J/K)

IC\textsubscript{50}  concentration of a compound that is required to achieve 50% inhibition *in vitro* (M)

IC\textsubscript{95}  concentration of a compound that is required to achieve 95% inhibition *in vitro* (M)

IC\textsubscript{99}  concentration of a compound that is required to achieve 99% inhibition *in vitro* (M)

$K_D$  equilibrium dissociation constant, $K_D = \frac{k_{\text{off}}}{k_{\text{on}}}$ (M)

$K_i$  inhibition constant, [I] leading to $\frac{V_{\text{max}}}{2}$ (M)

$K_{\text{app}}$  apparent Michaelis-Menten constant in presence of inhibitor (M)

$K_M$  Michaelis-Menten constant, [S] required to reach $\frac{V_{\text{max}}}{2}$ (M)

$k_{\text{off}}$  dissociation rate constant (s\textsuperscript{-1})

$k_{\text{on}}$  association rate constant (M\textsuperscript{-1}s\textsuperscript{-1})

$R$  ideal gas constant (J/mol K)

SI  selectivity index, SI = $\frac{\text{CC}_{50}}{\text{IC}_{50}}$ (unitless)

$T$  temperature (K)
\( v_i \) initial velocity of an enzymatic reaction (mol/s)

\( V_{\text{app}} \) apparent \( V_{\text{max}} \) in presence of inhibitor (mol/s)

\( V_{\text{max}} \) maximum velocity of an enzymatic reaction (mol/s)

**Units**

Å  Angstrom

°C  degree Celsius

h  hour

J  joule

K  kelvin (temperature)

kcal  kilocalorie

kDa  kilodalton

L  liter

µL  microliter

mol  mole, 1 mol = 6.022 × 10^{23} units of a chemical entity

M  molar, 1 mol per liter

mM  millimolar

µM  micromolar

nM  nanomolar

µm  micrometer

nm  nanometer

s  second

**Carbohydrates**

3'NANL  3'-N-acetylneuraminyllactose

DANA  Neu5Ac2en

Fuc  fucose

Gal  galactose

GalNAc  \( N \)-acetylgalactosamine

Glc  glucose

GlcNAc  \( N \)-acetylglucosamine
Man  mannose
MUN  2’-(4-methylumbelliferyl)-α-D-N-acetyleneuraminide
Neu5Ac  N-acetyleneuraminic acid
Neu5Ac2en  2-deoxy-2,3-didehydro-D-N-acetyleneuraminic acid
Neu5Gc  N-glycolyneuraminic acid

Amino acids
A, Ala  alanine
R, Arg  arginine
N, Asn  asparagine
D, Asp  aspartic acid
C, Cys  cysteine
E, Glu  glutamic acid
Q, Gln  glutamine
G, Gly  glycine
H, His  histidine
I, Ile  isoleucine
L, Leu  leucine
K, Lys  lysine
M, Met  methionine
F, Phe  phenylalanine
P, Pro  proline
U, Sec  selenocysteine
S, Ser  serine
T, Thr  threonine
W, Trp  tryptophane
Y, Tyr  tyrosine
V, Val  valine
Chapter 1

Introduction

1.1 Preamble

Each year, an estimated 55–60 million people die of various causes\(^1\). About a quarter of all these deaths originate from infectious diseases, pathologies caused by bacteria, fungi, parasites or viruses, that are often transmissible from human to human\(^{1,2}\). These numbers vary greatly depending on the country, and are the highest in developing and underdeveloped countries.

Viruses are a major cause of infectious diseases, and have been responsible for several serious pandemics throughout history. The Spanish H1N1 influenza pandemic of 1918 is undoubtedly the greatest of all, as a quarter of the population is believed to have been infected (about 500 million people), resulting in the death of 40–50 million\(^{3,4}\). Another contemporary pandemic is caused by the human immunodeficiency virus, HIV, the aetiological agent of acquired immunodeficiency disease syndrome (AIDS). According to the World Health Organisation (WHO), 78 million people have been infected by HIV since the beginning of the pandemic in 1981, and the cumulative AIDS-associated deaths amount to 39 million\(^{5,6}\). In 2013, 35 million people were still living with the virus and 1.5 million have died\(^7\).

No less than a third of all deaths attributed to infectious diseases occur in children under 5 years of age\(^1\). Among those, respiratory infections are prevalent, accounting for 18% of mortality among the paediatric population\(^8\). It is also the leading cause of infections in adults\(^9,10\). The pathogens associated with this disease burden are mostly
viruses, among which rhinovirus, human respiratory syncytial virus (hRSV), human parainfluenza viruses (hPIV), influenza virus and human metapneumovirus (hMPV) are the most prevalent\textsuperscript{10}. Parainfluenza viruses and hRSV are the leading cause of acute lower respiratory tract infections in infants, elderly and the immunocompromised worldwide.

Other important paediatric viral pathogens include enteroviruses, a genus of viruses responsible for a variety of illnesses in children\textsuperscript{11}. Some members like enterovirus 71 (EV71), causing hand, foot and mouth disease (HFMD), can induce severe pulmonary oedemas, encephalitis and meningitis, and are especially prevalent in Asia\textsuperscript{12–14}. From 2008 to 2012, there have been over 7 million reported cases of HFMD and over 2,400 deaths in China alone\textsuperscript{15}.

Despite the important health burden caused by hPIVs, hRSV and EV71, neither treatments nor vaccines are yet available. Effective novel drug candidates are therefore critically needed.

This Introduction will provide an overview of paramyxoviruses and enterovirus infections, with an in-depth description of human parainfluenza type-3 virus and human respiratory syncytial virus biology. It will also introduce some of the principals and methodologies of early-stage drug discovery, and the current status of small-molecules in development for the treatment of hPIV-3, hRSV and EV71 infections. Finally, the specific aims of this Thesis will be described.
## 1.2 Paramyxoviruses and Respiratory Infections

### 1.2.1 Taxonomy

Paramyxoviruses belong to the *Paramyxoviridae* family, itself deriving from the large *Mononegavirales* order. They are all enveloped, negative-sense single-stranded RNA viruses, and as such belong to the virus-group V according to the Baltimore classification.

*Paramyxoviridae* is one of the largest families of the order with *Rhabdoviridae*, and includes several ubiquitous human respiratory pathogens such as hPIVs and hRSV. The well-known mumps and measles viruses, causing paediatric diseases, are also part of the family. Paramyxoviruses are not all human pathogens. Viruses like Hendra and Nipah are highly lethal to horses and pigs respectively, and to humans by close-contact transmission from intermediary hosts. Newcastle disease virus (NDV) and some parainfluenza viruses can cause respiratory infections in birds, while Sendai virus is infectious towards rodents. Other families of the *Mononegavirales* order also comprise important human and animal zoonotic pathogens such as Ebola virus (*Filoviridae*), responsible for highly severe haemorrhagic fever, rabies virus (*Rhabdoviridae*) causing central nervous-system infection, or the neurotropic Borna disease virus (*Bornaviridae*).

*Paramyxoviridae* is divided into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae*, from which derive five and two genera respectively: *Avulavirus*, *Henipavirus*, *Morbillivirus*, *Respirovirus*, *Rubulavirus*; and *Pneumovirus*, *Metapneumovirus*. While the two antigenic subgroups A and B of hRSV belong to the *Pneumovirus* genus, hPIVs belong either to the *Respirovirus* genus (serotypes 1 and 3) or to the *Rubulavirus* genus (serotypes 2 and 4).

### 1.2.2 Epidemiology and pathogenesis

hRSV and hPIVs are the principal cause of acute lower respiratory tract infections (ALRI) in young children worldwide. hRSV is the main aetiological agent and is thought to be globally responsible for about 160,000 deaths per year among paediatric services, although disease often occurs as a result of co-infections with other respiratory pathogens. In developed countries, about 0.5 to 1% of RSV-infected infants are hospitalised. Among parainfluenza viruses, hPIV-1 and -2 are the main causative agents of
croup, while hPIV-4 causes mild respiratory infections and is rarely detected\textsuperscript{23,24}. hPIV-3, on the other hand, is the most virulent and can induce severe bronchiolitis and pneumonia\textsuperscript{10,25,26}. About 7\% of acute respiratory infections in the USA are caused by hPIV-1 and -3, which represents over 23,000 hospitalisations per year\textsuperscript{27}.

Nowadays, about a third of children are infected by either hPIVs or hRSV in the first year of life, and more than 90\% have been infected at least once before the age of 2\textsuperscript{28}. Infections can occur throughout the year, but they usually peak during winter and early spring months each year depending on the country\textsuperscript{23,29}.

Apart from being responsible for a large number of hospitalizations among infants, hRSV and hPIV-3 are a serious threat to the elderly, the immunocompromised and transplant patients\textsuperscript{23,30–32}. As an example, hPIV-3 has been reported to cause symptoms similar to those of H1N1 influenza virus infection in the elderly\textsuperscript{25}. Because the viruses induce a strong immune response upon infection, that is largely responsible for the severity of the symptoms, premature children as well as children with congenital heart disease are at high risk. Furthermore, patients infected by hRSV during the early years of life could be at risk of subsequent development of wheezing or asthma later in life\textsuperscript{23,33}. A third paramyxovirus, referred to as human metapneumovirus virus (hMPV), is often associated with hPIV and hRSV infections and is a cousin of hRSV from the Metapneumovirus genus\textsuperscript{34,35}. Like hRSV, it causes respiratory illnesses in infants and elderly but with lower severity.

These viruses incubate for typically two days up to a week in the nasopharyngeal epithelium before spreading to the lower respiratory tract, causing bronchiolitis and pneumonia. The infection of the airway epithelial cells leads to inflammation and necrosis, dead cells and mucus thus accumulating in the airway and provoking obstruction. Both hPIV-3 and hRSV infections induce an important immune response, more responsible for the high severity of the disease than cytopathy itself\textsuperscript{23,26}. Infants primarily infected with hRSV and hPIV-3 acquire a short-lasting immunity to these pathogens and can be repeatedly infected throughout life, although reinfections are often less severe.
### 1.2.3 Genome and proteome

The genome of paramyxoviruses is made of a single-stranded negative-sense genomic viral RNA (vRNA). Unlike positive-sense RNA viruses, whose RNA can be directly translated to proteins by the host cell machinery, negative-sense RNA needs to be transcribed to mRNA by the viral RNA-dependent RNA polymerase (RdRp) prior to translation. The genome is typically about 15,000 nucleotides (nt) long, with essential 40–70 nt leader and 20–160 nt trailer regions on its 3’ and 5’ ends respectively, and short < 200 nt intergenic regions (Figure 1.1, p. 5). Each gene is flanked by 3’ gene-start (GS) and 5’ gene-end (GE) sequences that dictate the beginning and end of the transcription of each gene. mRNAs are transcribed sequentially from 3’ to 5’ end of the vRNA and are less abundant as transcription moves downstream, due to the propensity of the RdRp to stop at intergenic regions. The amount of viral proteins synthesised in the cells is therefore proportional to their respective levels of mRNA.

![Figure 1.1: Genome-organisation of selected members of the Respirovirus, Rubulavirus, and Pneumovirus genera. Genes are represented by coloured rectangles, and the proteins they encode by the enclosed letter. The number under and above each gene represents its nucleotide length and associated protein amino-acid length respectively. The underlined numbers represent the nucleotide length of intergenic regions, or leader (le) and trailer (tr) lengths. Blue: non-structural and polymerase-associated protein, Yellow/Purple: matrix protein, Red: structural envelope protein, Brown: non-structural protein. Figure adapted from Fields Virology, 6th Edition.](image-url)
Chapter 1. Introduction

The genomic and proteomic structures of respiroviruses and rubulaviruses are fairly similar. They are composed of 6 genes, each coding for a single protein via translation of respective mRNA, to the exception of the non-structural C, D, V, I and W proteins depending on the virus. The C protein mRNA of respiroviruses is transcribed from a shifted open reading frame (ORF) from the P gene, while the D, V, W, I proteins are translated from an RNA-edited P mRNA. The proteins that are constitutively translated from the 3’ to the 5’ genomic ends for all parainfluenza viruses are: the nucleocapsid protein N, the phosphoprotein P, the matrix protein M, the fusion protein F, the haemagglutinin-neuraminidase HN, and the large RNA-dependent RNA polymerase L.

Table 1.1: Proteome characteristics of hPIV-3 and hRSV

<table>
<thead>
<tr>
<th>Category</th>
<th>Protein</th>
<th>Mass (kDa)</th>
<th>Identity (%)</th>
<th>Glycosylation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hPIV-3</td>
<td>hRSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structural</td>
<td>M</td>
<td>39.5</td>
<td>28.7</td>
<td>18.91</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>60</td>
<td>63.4</td>
<td>21.56</td>
</tr>
<tr>
<td></td>
<td>HN</td>
<td>64.2</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>–</td>
<td>32.6</td>
<td>&gt; 40</td>
</tr>
<tr>
<td></td>
<td>SH</td>
<td>–</td>
<td>7.5</td>
<td>1c</td>
</tr>
<tr>
<td>Nucleocapsid-associated</td>
<td>P</td>
<td>67.6</td>
<td>27.1</td>
<td>20.70</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>255.8</td>
<td>250.3</td>
<td>22.96</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>57.8</td>
<td>43.4</td>
<td>18.77</td>
</tr>
<tr>
<td></td>
<td>M2-1</td>
<td>–</td>
<td>22.1</td>
<td>–</td>
</tr>
<tr>
<td>Non-structural</td>
<td>M2-2</td>
<td>–</td>
<td>10.6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>NS1</td>
<td>–</td>
<td>15.5</td>
<td>–</td>
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<tr>
<td></td>
<td>NS2</td>
<td>–</td>
<td>14.7</td>
<td>–</td>
</tr>
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<td></td>
<td>C</td>
<td>23.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Dd</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\(a\) Mass values and glycosylation sites were taken from the UniProt database, for hPIV-3 Wash/47885/57 (taxon 11217) and hRSV-A2 (taxon 11259).

\(b\) Protein identities were calculated using the Clustal Omega program\textsuperscript{36}.

\(c\) Un-glycosylated form is dominant in cells\textsuperscript{37}

\(d\) No data available

The hRSV genome is composed of more genes, 10 in total, organised in a slightly different way and coding for 11 proteins from 10 mRNAs: the non-structural proteins NS1 and NS2, the nucleocapsid protein N, the phosphoprotein P, the matrix protein M, the short-hydrophobic protein SH, the glycoprotein G, the fusion protein F, the matrix-
associated proteins M2-1 and M2-2, and the large RNA-dependent RNA polymerase L. M2-1 and M2-2 are encoded by 2 distinct but slightly overlapping reading frames on the M2 mRNA.

Proteome differences and similarities between hPIV-3 and hRSV specifically are summarised in Table 1.1, p. 6. The two viruses have 5 proteins in common, sharing similar functions: M, F, P, L and N. While their size is in the same range, the relative percentage of identity of their amino acid sequence differs significantly. It is in fact below 30% for all proteins and they are therefore not considered homologous. Their function is however alike, as it will be detailed in Section 1.2.5, p. 8. Two of those common proteins are envelope proteins (M, F), while the other three are nucleocapsid-associated (P, L, N).

1.2.4 Structure

Paramyxoviruses are enveloped pleomorphic virus particles, hPIV-3 measuring about 150–250 nm in diameter. The hRSV diameter is more variable (80–350 nm), as it can be spherical or filamentous. In the latter form, found mostly in culture, hRSV can be up to 10 µm long and 60-200 nm wide.

The Figure 1.2 (p. 8) illustrates the structure of hPIV-3 and hRSV virus particles. The envelope of paramyxoviruses is made of a phospholipid bilayer taken up from the plasma-membrane of the host cell during the budding process. Virions appear as covered with surface spikes on electron micrographs (Figure 1.2, right), that are in fact the envelope structural glycoproteins – F and HN for hPIV-3; F, G and SH for hRSV – incorporated into the bilayer via their N-terminus transmembrane domains. The matrix protein M, essential for virus integrity\textsuperscript{38,39}, shapes the envelope of the virus by binding to the structural glycoproteins, the lipid bilayer and the nucleocapsid. In the case of hRSV, it can also bind RNA\textsuperscript{40}. The nucleocapsid is helical-shaped, composed of genomic and antigenomic RNA, N, P and L for all paramyxoviruses, and is associated with virus-specific non-structural proteins such as M2-1 in the case of hRSV\textsuperscript{16,41}. The shape of the nucleocapsid is mediated by the N protein, which helps packaging the genome tightly, thus stabilising it\textsuperscript{42–44}.
1.2.5 Replication cycle of hPIV-3 and hRSV

1.2.5.1 Cell recognition

To initiate host cell infection, hPIV-3 and hRSV bind to cellular receptors of the upper respiratory tract principally due to engagement with HN and F/G, respectively. HN recognises glycoconjugates that carry a terminal N-acetylneuraminic acid (Neu5Ac, see structure on Figure 1.141, p. 48) linked through an α2-6 or α2-3 bond \(^{46-48}\) (see Section 1.2.8, p. 14). hPIV-3 has also been shown to bind heparan sulfate presented on cell surfaces \(^{49}\). On the other hand, hRSV F and G target receptors containing heavily sulfated glycosaminoglycans such as chondroitin sulfate or heparan sulfate \(^{50,51}\). The envelope structural protein SH is not involved with host cell recognition by hRSV. It is non-essential for viral replication, although it plays a role in pathogenesis severity \(\textit{in vivo}\) \(^{52,53}\). It acts as a hydrophobic transmembrane viroporin ion channel, and as such destabilises host cells by permeabilising their membranes through self-oligomerisation as a pentamer at the plasma membrane \(^{54,55}\).

1.2.5.2 Fusion

Upon binding to the receptor, the virus enters the cell by F-mediated fusion of the viral envelope with the plasma membrane. Both hPIV-3 and hRSV fuse directly with the membrane, but hRSV also enters the cell via clathrin-mediated endocytosis \(^{56}\) or micropinocytosis \(^{57}\) followed by fusion with the endosomal membrane. Paramyxoviruses F proteins
are type-I fusion proteins (containing mostly $\alpha$-helices, and are trigger-activated\(^{58}\)). Before they become active, they are cleaved from precursor $F_0$ by proteolytic cleavage by a furin-like protease into $F_1$ and $F_2$, two subunits linked by a disulfide bond. $F_1$ and $F_2$ are then presented as a trimeric precursor form on the viral envelope. A hydrophobic fusion peptide, located at the N-terminus of $F_1$, inserts into the host cell membrane and triggers fusion. The precise manner in which fusion is activated in hRSV infection is unclear. In hPIV-3 infection, however, HN has been shown to both stabilise $F_0$ into its pre-triggered state\(^{59}\), and to activate it upon receptor binding\(^{60}\). Also, F and HN must be of the same serotype for fusion to take place\(^{61}\) (see Section 1.2.8, p. 14).

F is not only responsible for fusion of the viral envelope with the host cell membrane; once transported to the cell surface it can also mediate fusion with neighbouring cells. As shown in Figure 1.3, this fusion results in formation of large syncytia containing several nuclei, a feature especially characteristic of hRSV infection but also observed in hPIV-3 infection.

![Figure 1.3: Syncytium formation during hPIV-3 and hRSV infection in vitro](image)

Figure 1.3: Syncytium formation during hPIV-3 and hRSV infection in vitro. Left: hPIV-3 infection of HeLa cells. Right: hRSV infection of HEp-2 cells. The white arrows point towards sites of syncytium formation. Figures adapted from Porotto et al.\(^{62}\) and Domachowske et al.\(^{63}\).

### 1.2.5.3 Genome transcription and replication

Once fusion has occurred, the nucleocapsid is released into the cytoplasm. There, the RdRp transcribes the negative-strand vRNA into individual mRNAs, ready to be translated to viral proteins by the host cell machinery. The vRNA is concurrently replicated.
into positive-sense antigenomic RNA, which in turn serves as a template for the synthesis of genomic RNA. During this process, the RdRp disregards any GS/GE sequences and intergenic regions. The protein L bears the RdRp function, and is associated with vRNA, P and N (hPIV-3), or vRNA, P, N and M2-1 (hRSV). N associates with the genome, and forms a helical nucleocapsid that becomes resistant to RNAses. The heavily phosphorylated P protein stabilises the nucleocapsid and facilitates its solubility\(^\text{64}\), while it also serves as a co-factor for L\(^\text{65}\) and helps with the binding of L to N. The M2-1 protein is an important processivity factor of hRSV transcription, and its function will be further detailed in Section 1.2.9, p. 21. It binds both P and RNA in a semi-competitive manner to prevent early termination of transcription\(^\text{66,67}\). The M2-2 protein regulates the balance between vRNA replication and transcription. Viruses that lack M2-2 have been shown to have higher levels of mRNA as compared to genomic/antigenomic RNA. It promotes vRNA replication and inhibits transcription in a dose-dependent manner\(^\text{68–70}\).

### 1.2.5.4 Protein synthesis and maturation

Protein synthesis from mature viral mRNAs is carried out by the host cell machinery, in the endoplasmic reticulum (ER). While most mRNAs serve as templates for the synthesis of single proteins, both viruses possess polycistronic mRNAs from which several proteins can be synthesised. The M2-1 and M2-2 proteins of hRSV are synthesized from the M2 mRNA, which contains 2 ORFs. M2-1 is synthesised upstream of M2-2, whose ORF slightly overlaps the 5’ end of one of M2-1. Additionally, another form of G is synthesised from the G mRNA, but from a slightly shifted ORF that initiates translation within the N-terminal envelope-anchored domain of G. This shortened version of G is soluble and secreted by the virion, and present in large amounts in infected cells\(^\text{71}\). It is involved in modulating the host-response to infection\(^\text{72–74}\). hPIV-3 D and C proteins are translated from the P mRNA. C is encoded on an ORF-shift of the P gene, and is involved in inhibition of infection \textit{in vitro} as well as inhibition of the host interferon response\(^\text{75,76}\). D, whose function is unclear, is translated from an edited mRNA where a G residue was added to the upstream 5’end of the P ORF, resulting in a shorter mRNA\(^\text{77,78}\). The V protein is translated via a similar mechanism, although its existence is poorly documented.
As discussed in Section 1.2.5.2 (p. 8), F is activated from the inactive form F\(_0\) to the active form F\(_1\)/F\(_2\) by a furin-like protease while travelling through the trans-Golgi network. The protease recognises specific sequences within F\(_0\), and as a general rule cleaves after a R-X-R/K-R motif: RTKR, and RARR or KKRKRR for hPIV-3\(^{79}\) and hRSV\(^{80}\) respectively.

The structural proteins F, HN, SH and G are transported to the Golgi apparatus, where they undergo post-translational glycosylation. As summarised in Table 1.1 (p. 6), HN possesses 3 N-linked glycans that appear to be essential for receptor recognition as well as fusion activation\(^{81}\) (detailed in Section 1.2.8, p. 14). hPIV-3 F is N-glycosylated on 3 residues, compared with 5 for hRSV. SH can be glycosylated at one site and phosphorylated at another, but the un-modified form is the most abundant in cells\(^{37}\). G is the most heavily glycosylated, with 3–5 N-glycans and over 40 O-glycans\(^{82}\). These glycosylations typically involve the transfer of GlcNAc\(_2\)-Man\(_n\)-Glc\(_a\)\(^a\) to the amide group of specific arginines during polypeptide elongation in the ER lumen (N-glycosylation), or N-acetylgalactosamine (GalNAc) bound to the hydroxyl group of most serines or threonines at a later stage in the Golgi apparatus (O-glycosylation).

Lastly, a number of proteins undergo a series of important phosphorylation events mainly on non-O-glycosylated serine and threonine residues. This is especially the case for the hRSV P protein, whose dynamic reversible phosphorylation is critical to maintain its function and interaction with M2-1 on the one hand\(^{65,83}\), and to ensure the integrity of virus particles on the other hand\(^{84}\). M2-1 phosphorylation at serines 58 and 61 are critical for its anti-termination activity\(^{67,85}\). This process is reversible, and both the phosphorylated and unphosphorylated forms of M2-1 can be found in infected cells. Lastly, hPIV-3 P phosphorylation is required for efficient viral replication\(^{86}\), while C protein phosphorylation seems to be important for infection inhibition\(^{87}\).

### 1.2.5.5 Virus assembly and release

Following protein synthesis, the glycosylated structural envelope proteins are transported to the plasma membrane of infected cells through the exocytic pathway. The matrix protein M binds the nucleocapsids, and mediates their transport to the assembly site. In hRSV infection, this binding takes place in inclusion bodies and M2-1 acts as an inter-

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\(^a\)GlcNAc: N-acetylglucosamine, Man: mannose, Glc: glucose, Gal: galactose
mediary between M and the nucleocapsid\textsuperscript{88,89}. At the assembly site, M binds the inner part of the plasma membrane tightly, as well as the cytoplasmic tails of the envelope glycoproteins\textsuperscript{90,91}. This process is believed to happen at lipid-raft domains, which could mediate the segregation and clustering of G, HN, F and SH\textsuperscript{88,92}. The M protein is critical to the production of filamentous forms of hRSV\textsuperscript{39}. In the case of hPIV-3, the sole expression of M in absence of other viral proteins has been shown to be sufficient for the formation of hPIV-3 virus-like particles (VLP)\textsuperscript{93}. Finally, upon recruitment of all partners M helps shaping the budding particles by curving the plasma membrane of the cell, and enveloped virus progeny are formed.

In hPIV-3 infection, HN facilitates the release of newly synthesised virions by cleaving off the terminal sialic acid of glycoconjugate-containing cellular receptors present at the surface of the infected and neighbouring cells (see Section 1.2.8, p. 14). This process facilitates viral propagation, and at the same time prevents the accumulation of virus particles at the cell surface. Interestingly, most of hRSV particles remain bound to the cell upon budding\textsuperscript{35}.

1.2.5.6 Role of hRSV non-structural proteins

The non-structural NS1 and NS2 proteins are not packaged in hRSV. They are small proteins of about 15 kDa produced in the cell during infection. As their gene is located on the 3’ end of hRSV genome, they are produced in substantial amounts (see Section 1.2.3, p. 5). NS1 and NS2 are known inhibitors of interferon (IFN) I and II induction and signalling\textsuperscript{94–96}. They have also been shown to suppress apoptosis\textsuperscript{97}. The proteins can bind each other, and are typically found in the nucleus (NS1) or the mitochondria (NS2, and NS1/NS2 complex)\textsuperscript{98}.

1.2.6 Immunogenicity

As mentioned briefly in Section 1.2.5 (p. 8), several viral proteins of hPIV-3 and hRSV have the capacity to modulate the host cell immune response to infection. What triggers antibody production, however, are the envelope glycoproteins F, G in the case of hRSV, and F, HN in the case of hPIV-3. Each of these glycoproteins bear several antigenic sites.
1.2. Paramyxoviruses and Respiratory Infections

1.2.6.1 hPIV-3

HN and F are the only hPIV-3 antigens that generate neutralising antibodies. Among them, anti-HN antibodies are more protective than anti-F antibodies. This is partly explained by the fact that HN is a protein that can regulate F activity. Neutralising anti-HN antibodies can thus also block fusion and syncytium formation by indirectly inhibiting F function. HN possesses 6 antigenic sites, all located in its globular domain. Monoclonal antibodies (MAb) binding to three of them (A, B and C, containing 11 epitopes) have been shown to neutralise infection by targeting haemagglutinin or neuraminidase activity, while MAb binding to the other 3 (D, E, F) have no such activity. The F protein, on the other hand, possesses 3 neutralising antigenic sites (divided in 14 epitopes), and 4 non-neutralising antigenic sites (divided in 6 epitopes). Together, neutralising anti-hPIV-3 -F and -HN antibodies show very little cross-reactivity with other parainfluenza subtypes.

1.2.6.2 hRSV

There are 2 antigenic subgroups of hRSV, hRSV-A and hRSV-B, sharing about 81% identity. They both cause disease, although hRSV-A is more frequently detected and often more virulent than hRSV-B. Similar to hPIV-3, the F and G proteins are the only hRSV antigens generating neutralizing antibodies. However in the case of hRSV, anti-F antibodies are more protective than anti-G antibodies. They are also more protective against the different subgroups of hRSV, as F proteins are generally more conserved than G. There are 5 antigenic sites on F, predominantly located on the F1 subunit and from which neutralising antibodies can inhibit fusion. For antibodies to recognise binding epitopes, it has been shown that F needs to be properly folded. The antigenicity of the G protein is very variable between hRSV isolates, and is used to segregate hRSV into the two antigenic subgroups A and B. There are 3 categories of epitopes on the protein G that can be recognised by MAb: those common to all subgroups, where the glycosylation state of G is not important, those that are group-specific, and those that are strain-specific, where G is usually glycosylated. Anti-G antibodies do not have strong neutralising properties, and can bind folded as well as linear epitopes on the G protein.
1.2.7 Prevalent animal models of infection

A variety of animals are used as models of hPIV-3 and hRSV infection24,110,111. The mouse model, commonly used in pre-clinical studies of many drug candidates, is not very permissive to hPIV-3 and hRSV infection. This is especially the case for hPIV-3, and a chimeric murine Sendai virus bearing hPIV-3 HN and F must be used to infect mice since it is the natural host for Sendai virus, which is closely related to hPIV-3112,113. hRSV, however, can directly infect mice although the permissiveness of cotton rats to hRSV infection is greater than of mice111. The cotton rat is also a better small animal model of hPIV-3 infection, since it can be infected with wild-type strains of the virus114. These murine models are relatively easy to handle given their small size, and are good molecular tools due to the high genetic diversity available to laboratories. However, they are genetically remote from humans, and infection by hPIV-3 and hRSV in these animal models is mostly asymptomatic. Unlike for influenza research115, ferrets are not widely used as models of infection for hPIV-3 and hRSV. Lambs, however, are a good model of neonatal hRSV infection as they are moderately permissive and develop a pathology similar that in hRSV-infected infants116.

Finally, non-human primates are the best models for both viruses, as they are genetically and morphologically close to humans and show clinical signs of infection similar to those in humans. Chimpanzees, rhesus macaques and African green monkeys are all good models of hPIV-3 and hRSV infection, and are mostly used in the pre-clinical evaluation of vaccines53,117–122. The major disadvantages in using these animal models are the associated cost, requirement of specialised facilities and technicians, and ethical concerns.

1.2.8 hPIV-3 haemagglutinin-neuraminidase

As it has been mentioned previously, HN is involved in 4 key steps of the viral replication cycle: entry, fusion stabilisation and triggering, and release. This section will review in detail the structure and function of HN, a protein essential to hPIV-3 replication.
1.2.8.1 Structural and physical properties

**Structure**  HN is a type-II transmembrane glycoprotein composed of an N-terminal intra-virion domain, followed by a short transmembrane domain, a cytoplasmic stalk and a C-terminal extracellular globular head of about 56 kDa. A schematic representation of the topology of HN domains and their respective size can be found in Figure 1.4, p. 16. As its name indicates HN is both a haemagglutinin and a neuraminidase, activities borne by its globular domain. The crystal structure of hPIV-3 HN globular domain was solved in 2004 by Lawrence and colleagues\(^ \text{123} \), at an atomic resolution of 2 Å. They characterised the structure in its apo-form, in complex with Neu5Ac, 2-deoxy-2,3-didehydro-D-\(-N\)-acetylneuraminic acid (DANA or Neu5Ac2en, an early inhibitor of neuraminidases), and the potent influenza virus sialidase inhibitor, zanamivir (see Figure 1.14, p. 48 for the structures). HN is a biologically active, calcium and pH-dependent dimeric six-blade \(\beta\)-propeller protein that associates to form homotetramers via disulfide bonding\(^ {123-125} \). Haemagglutination is optimal at 37 °C and pH 7, but still functions at 4 °C. The neuraminidase activity, on the other hand, is highly pH-dependent and optimal at pH 4.6, 37 °C, but is abolished at 4 °C. While the dual function of HN was known for a long time, the crystal structures provided structural proof that the protein possesses a single binding site mediating the dual function of sialic acid recognition and cleavage.

**Primary binding site**  HN’s primary binding site is a flexible cavity, suggesting that it could physically be modulated upon binding to sialylglycoconjugates to initiate receptor cleavage\(^ {123} \). The most flexible part of the binding site is a loop composed of the residues 210–221, so called the D216 loop. Recently, Winger and von Itzstein have demonstrated by molecular dynamics simulations that HN D216 loop is open in its apo-form, and that it closes upon receptor binding to better lock it in\(^ {126} \). This also suggests that HN could bind oligomers larger than sialic acid alone, as it has been proposed in the past\(^ {47} \).

The amino acids critical to the catalytic activity of neuraminidases are shared between several species, and involve an arginine triad, glutamic acid, aspartic acid and tyrosine residues. For hPIV-3 they are R192, R424, R502, E409, E549, D216 and Y530\(^ {123} \). Other amino acids have been reported to be important for HN activity, such as threonine 193, buried on the side of the cavity\(^ {123} \). When substituted to alanine\(^ {60,127,128} \) or isoleucine\(^ {129} \), it
Figure 1.4: The topology of hPIV-3 HN domains and structure. A. Schematic representation of the topology of hPIV-3 HN domains. Numbers at the top represent the amino acid length of each domain. IV: intra-virion domain, TM: transmembrane domain. B. Ribbon representation of the hPIV-3 HN dimer bound to zanamivir (in stick representation). The different colours outline the 6 blades of the β-propeller. C. Close-up of the primary binding site in complex with zanamivir. The 216-loop is coloured in orange. D. Surface representation of the primary binding site in complex with zanamivir. The models were created using PyMOL 1.7 (Schrödinger, LLC), and the PDB structure 1V3E.
was shown to increase HN receptor avidity and catalytic activity. The T193I HN mutant also becomes resistant to zanamivir. Finally, if HN is mutated at the D216 position the protein loses its neuraminidase activity (D216N or D216R). This could be due to a change in the D216 loop flexibility, as discussed above.

**Glycosylation**  
HN can be theoretically glycosylated with 4 N-linked glycans at asparagine residues N308, N351, N485 and N523. While glycosylation of N308, N351 and N523 has been observed in crystal structures, there is no evidence of N485 glycosylation at the present time. N-glycosylation typically occurs on asparagines included in a Asn-X-Ser/Thr motif, but N485 is followed by a proline which prevents successful glycosylation of the residue. The exact nature of HN’s N-linked glycans is however unknown, but they most likely contain mannose oligomers branching from N-acetyllactosamine. N-glycosylations appear to be important for HN haemagglutinin activity and fusion promotion, but not for HN interaction with F. A lower glycosylation level on HN appears to reduce virus haemagglutination and fusion.

**1.2.8.2 Receptor recognition and cleavage**

**Specificity**  
As a haemagglutinin, HN has the capacity to bind and aggregate red blood cells. In the specific context of a host cell infection however, it binds to cell-surface glycoconjugates containing a terminal sialic acid bound to N-acetyllactosamine (Galβ1-4GlcNAc; Gal: galactose, GlcNAc: N-acetylglosamnine) via an α2-3 or α2-6 linkage. Therefore, hPIV-3 HN recognises the sialyl-Lewis motif as a minimal glycan structure (Figure 1.5, p. 18). Unlike hPIV-1 HN that only recognises α2-3-linked sialic acids for instance, hPIV-3 HN can also bind N-glycolyneuraminic acid (Neu5Gc) when presented on the I histo-blood group antigen. The avidity of hPIV-3 HN for glycoconjugates is notably higher as they display more branched Neu5Ac-N-acetyllactosamine or contain more N-acetyllactosamine repeats in their core structure. HN also tends to have more affinity for sulfated or fucosylated glycans if they are not branched or relatively short.

As a neuraminidase, hPIV-3 HN removes terminal Neu5Ac residues from cellular glycoconjugates. Some evidence suggests that HN binds Neu5Gc, but there is no report of its cleavage. The reaction takes place at 37 °C, at an optimal pH of 4.6. Unlike
influenza virus neuraminidase, the hPIV-3 HN catalytic reaction has not been characterised although HN active site is composed of similar key residues. The substrate specificity of hPIV-3 HN is poorly documented. HN can cleave α2-3 or α2-6-linked sialic acids from galactose bound to GlcNAc via a β1-3 or β1-4 linkage, but whether it has more affinity for α2-3 or α2-6 sialic acid is unknown. HN is able to metabolise the substrate MUN (2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid) to release the fluorophore 4-methylumbelliferone, but it is unable to metabolise the hPIV-1 and hPIV-2 substrate 3′NANL (3′-N-acetylneuraminyllactose).

The data available to date on the sialylglycan recognition and cleavage specificities of HN is fairly limited, and mostly based on glycan array experiments where glycan libraries are fixed to a solid surface via linker sets of variable lengths. The glycans’ presentation to HN do therefore not necessarily reflect how they are presented in a biological context. While they have provided clues towards an understanding of what glycan structures can bind HN, the full extent of the receptors, proteins and/or lipid conjugates involved remains unknown. In addition, Fukushima and colleagues have recently demonstrated that hPIV-3 has the ability of infecting cells that present α2-6-linked sialic acids only as potently as normal cells, suggesting that HN could potentially bind a wide array of α2-6-linked sialylglycoconjugates. Such glycans have not yet been extensively studied.

Figure 1.5: Known sialylglycan epitopes recognised by hPIV-3 HN. a. Sialyl-LewisX motif. b. I histo-blood group antigen motif.
Secondary binding sites  Although most of glycan recognition appears to be mediated by the HN primary active site, two secondary binding sites have been identified on HN. One of them is located at the dimer interface, around residues N551 and H552, and close to the primary binding site (about 15–20 Å). An asparagine to aspartic acid substitution at position 551 (N551D) does not change receptor avidity, but increases HN-mediated activation of F, and fusion (see next Section). A histidine to glutamine substitution at position 552 on the other hand (H552Q, occurring in nature), increases HN's avidity for sialylglycoconjugates and makes the protein resistant to zanamivir binding but not to DANA binding on site II, partly due to zanamivir's bulky guanidino group. In other words, when zanamivir binds the primary site, Neu5Ac and DANA are able to bind the site II. Zanamivir is however able to bind the site II of the N551D variant should the primary site be occupied.

Mishin and colleagues have characterised another potential binding site near residue N523, masked by the N-glycosylation at this position. In their study, they showed that an asparagine replaced by an aspartatic acid at position 523, resulting in a loss of glycosylation, increased the avidity of HN for red blood cells receptors while the neuraminidase activity remained unchanged.

![Figure 1.6: Positions of hPIV-3 HN secondary binding sites. Surface representation of HN, where the secondary binding sites at the dimer interface and at position 523 are coloured in cyan and purple respectively. The model was created using PyMOL 1.7 (Schrödinger, LLC), and the PDB structure 1V3E.](image-url)
1.2.8.3 Involvement in fusion

HN has a dual role with the F protein: it contributes to its stabilisation prior to fusion, and it mediates fusion activation. These 2 functions of HN are intertwined, but take place at different times of infection.

**Fusion stabilisation** The stabilisation of F in its pre-triggered state is mediated by the HN cytoplasmic stalk\(^{59,127,136,137}\). HN needs to be engaged with the cellular receptor for fusion to occur, but fusion can happen without HN although it is less efficient. There is however more fusion when HN is absent than when its receptor binding activity is blocked by zanamivir, suggesting that HN stabilises F before fusion occurs, independently of HN receptor binding and cleavage functions\(^{59,125}\). Without HN, F is not stabilised prior to fusion and is displayed in its post-fusion state, as fusion would already have been triggered.

**Fusion triggering** The dimerisation of HN as well as its secondary binding site at the dimer interface appear to be largely responsible for hPIV-3 fusion activation and efficiency, as they intimately regulate the behaviour of the cytoplasmic stalk\(^{59,60,127,128,132,136–138}\). HN binds F during the whole process of fusion, whether it be for the stabilisation of F or fusion triggering. While HN binding to its receptor activates fusion, its neuraminidase activity has an antagonistic effect on fusion\(^{127}\). Neuraminidase-inactive HN can however promote fusion, suggesting that these events are not so strongly related to HN enzymatic activity\(^{130}\). Naturally-occurring HN N551D mutants, able to bind zanamivir on their site II, have enhanced F activation and fusogenicity\(^{128}\). H552Q mutants at HN dimer interface, that have a high affinity for sialylglycoconjugates and better dimeric stability, yield hyper-fusogenic virus particles that become non-infectious in differentiated primary human airway epithelial cells (HAE)\(^{60,125}\). H552Q combined with Q559R, a mutation that impairs triggering of fusion, decreases HN interaction with F and fusion activation, as well as HN dimer formation. Interestingly, these variants yield infectious particles in HAE\(^{132,138}\).

It is believed that when HN has a higher rate of neuraminidase activity than receptor binding, a loss fusion occurs because HN is in contact with F for a shorter amount of
It is also speculated that highly fusogenic hPIV-3 variants could activate their fusion prematurely, resulting in non-infectious particles and a loss of fitness in lung tissues\textsuperscript{132,138–140}.

### 1.2.8.4 Regulation of HN function

HN is very much pH-dependent. The neuraminidase activity needs an acidic pH to be optimally triggered, but haemagglutination can occur at low and neutral pH. There exists a delicate balance between the catalytic and binding functions of the protein, that are not yet fully understood. Tappert and colleagues have conducted binding and enzymatic studies on HN and suggest that HN is regulated by the density of sialic acid on cell surfaces\textsuperscript{141}. They conclude that a high concentration of sialic acid at the surface of a cell would drive the local pH to low values, promoting HN sialidase activity. A low concentration of sialic acid, on the other hand, they postulate would keep the local pH at neutral values thus favouring receptor recognition. As a consequence, they state that HN is a neuraminidase able to hold on to its substrate for long periods of time, and that the activation of the neuraminidase at high densities of Neu5Ac can prevent excessive fusion. This postulate is in accordance with Palmer and colleagues who demonstrate that excessive fusion leads to the production of non-infectious virus progeny\textsuperscript{139}, and low densities of sialic acid are in favour of a better controlled fusion as HN can interact and stabilise F for longer periods of time.

In summary, and as described above, the haemagglutinin-neuramindase plays a critical role in 4 major events in the life cycle of hPIV-3. These essential features of the protein make it a target of choice for the discovery of anti-parainfluenza viral agents.

### 1.2.9 hRSV M2-1

Human respiratory syncytial virus (hRSV) processivity factor and anti-terminator M2-1 is a protein arousing more and more interest in the field of hRSV research. M2-1 is required for the virus to process vRNA efficiently, by preventing early interruption of viral transcription.
1.2.9.1 Structure

The structure of M2-1 was solved by solution Nuclear Magnetic Resonance spectroscopy (NMR) and crystallography; it is a 22 kDa, 194 amino acid α-helical protein.\textsuperscript{66,67,142} It is exclusively tetrameric \textit{in vitro} and \textit{in vivo}, and its oligomerisation state is required for its function. As shown on Figure 1.7, M2-1 is composed of a C-terminal α-helical core, a tetramerisation α-helix and an N-terminal Cys3-His1 zinc-binding domain. It possesses a particularly flexible region, located between the tetramerisation helix and the core domain. The zinc-binding domain is important for the function of M2-1 and the stability of the tetramer\textsuperscript{67,143}, and can be inhibited by small molecules\textsuperscript{144}.

![Figure 1.7: Structure of hRSV M2-1 protein. A, B, C. Surface representations of the tetramer. A. Side view. B. Top, C-terminal view. C. Position of a monomer relative to the tetramer. D. Surface representation of a monomer. E. Cartoon representation of a monomer. F. Ribbon representation of a monomer with regards to flexibility (B-factor). The thicker the ribbon, the more flexible the backbone region. RNA and P-binding regions are coloured in red and blue respectively. The region binding both P and RNA is coloured in pink. Serines 58 and 61 are coloured in yellow. The green sphere in E. and F. represents a zinc atom. The models were created using PyMOL 1.7 (Schrödinger, LLC), and the PDB structure 4C3B.](image-url)
1.2.9.2 M2-1 functions

In all virions, M2-1 is located between the matrix protein and the RdRp, close to the inner surface of the particle\(^{88,89}\). It also binds N, an interaction facilitated by the presence of RNA\(^{85}\). In cells, M2-1 co-localises in inclusion bodies together with other members of the RdRp\(^{66,145}\).

Unlike hMPV which does not need M2-1 to replicate\(^{146}\), hRSV M2-1 is essential for its replication. The levels of transcription of M2-1-deficient hRSV are significantly lower than those of wild-type counterparts\(^{142}\). As hRSV anti-termination factor, M2-1 prevents the early termination of transcription by the RdRp\(^{147}\). It is also a processivity factor of the polymerase that enhances the transcription of polycistronic mRNAs\(^{148}\). M2-1 also plays a role in nuclear factor κB (NF-κB) activation by interacting with the protein Rel A, a member of the NF-κB family\(^{149}\). In doing so, M2-1 could contribute to the pro-inflammatory response to hRSV infection.

1.2.9.3 M2-1 binding to P and RNA

The anti-termination activity of M2-1 is mediated by its semi-competitive binding to P and viral mRNA\(^{66,67,142}\). It has a high affinity for A-rich, mRNA-sense GE sequences, however M2-1 can bind 3' and 5' ends of hRSV genes as well as intragenic regions\(^{66,67,85,142,148,150}\). The binding to P occurs at a 1:1 stoichiometry and induces a conformational change of P and possibly M2-1\(^{151}\). P is also tetrameric, and interacts with the tetrameric M2-1 with a K\(_D\) \(\sim\) 8 nM\(^{151}\). Importantly, a physical interaction of M2-1 and P is required for anti-termination to occur\(^{65}\).

The P and RNA binding regions on M2-1 are distinct, although slightly overlapping (hence the semi-competitive binding). They were mapped by selectively mutating amino acids of the core domain, and analysing the capacity of M2-1 to bind RNA, P, and to process transcription using a minigenome system\(^{66,67}\). The minigenome uses cells transfected with plasmids encoding P, L, N and M2-1, together with a subgenomic replicon plasmid whose transcription of a luciferase gene is dependent on a fully functional P/L/N/M2-1 replication complex\(^{66,148}\). Table 1.2 (p. 24) reports the residues most important to M2-1, and the effect associated with their mutation. As shown in Table 1.2, M2-1
binding to P and RNA is essential to its anti-termination activity. The P and RNA binding regions of M2-1 can be visualised in Figure 1.7.

Table 1.2: Effect of selected mutations on amino acids critical for M2-1 function$^{66,67}$.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Inhibition</th>
<th>RNA binding</th>
<th>P binding</th>
<th>Inclusion bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minigenone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>A</td>
<td>A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S58</td>
<td>A/D</td>
<td>D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S61</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K92</td>
<td>D</td>
<td>D</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>K150</td>
<td>D</td>
<td>D/A</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>R151</td>
<td>D</td>
<td>D/A</td>
<td>D/A</td>
<td>D</td>
</tr>
<tr>
<td>K159$^b$</td>
<td>-</td>
<td>A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R126</td>
<td>D</td>
<td>-</td>
<td>D</td>
<td>-</td>
</tr>
<tr>
<td>T130</td>
<td>D</td>
<td>-</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>L148</td>
<td>A</td>
<td>-</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>T160</td>
<td>D</td>
<td>-</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>K162</td>
<td>-</td>
<td>-</td>
<td>D</td>
<td>-</td>
</tr>
<tr>
<td>N163</td>
<td>A/D</td>
<td>-</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

$^a$Double mutants.

$^b$Present in both RNA and P binding region.

1.2.9.4 Phosphorylation

Among the list of key residues the serines 58 and 61, that can be phosphorylated to phosphoserines by casein kinase I. The phosphorylation of S61 has been shown to be dependent on the phosphorylation of S58$^{85}$. They are located in the most flexible region of M2-1 (Figure 1.7, p. 22), in the vicinity of the RNA binding site, and play an important role in protein function$^{66,67,85,142,143}$. Phosphomimetic mutants, with serines 58 and 61 substituted by aspartic acid, have a structure similar that of a wild-type M2-1 but they are more negatively charged$^{67}$. The phosphorylation state of S58 and S61 seems to have no effect on M2-1/P binding, but it prevents the formation of M2-1/RNA complexes and it impairs M2-1 processivity$^{67,152}$. Interestingly, S58 and S61 mutated with neutral alanines have the same deleterious effect. The phosphorylation of M2-1 is transient, and both phosphorylated and unphosphorylated forms can be detected in cells$^{67,143}$. It is therefore speculated that a mix of both populations is needed for M2-1 to properly function.
1.3 Enterovirus 71 and Hand, Foot and Mouth Disease

Unlike hPIV-3 and hRSV, enterovirus 71 is not a paramyxovirus. It is a positive-sense single-stranded RNA virus, member of the *Enterovirus A* species, from the *Enterovirus* genus and the *Picornaviridae* family. This genus is the largest of the *Picornaviridae* family, with 7 human species and over 90 human serotypes. They include viruses such as human rhinovirus, responsible for common cold, human poliovirus, which can induce severe poliomyelitis (a paralytic disease commonly referred-to as “polio”), as well as other non-polio viruses such as human echovirus, Coxsackie virus and enterovirus, responsible for a wide range of mild-infections that can develop to respiratory illness, herpangina, hand foot and mouth disease (HFMD) and sometimes to severe neurological diseases such as meningitis and encephalitis\(^{153–156}\).

1.3.1 Hand, foot and mouth disease

HFMD is a disease principally caused by Coxsackie virus A16 (CV-A16) and enterovirus 71 (EV71), and is especially prevalent in south-east Asia and China. It mainly affects children under 5 years of age, and manifests itself as rashes on the hands, feet and mouth of patients. While infections are usually benign, the viruses can in some cases invade the central nervous system (CNS) and disseminate to the brain, and under such circumstances meningitis, encephalitis and flaccid paralysis can occur\(^{15,153,156–159}\).

EV71 alone has been responsible for several outbreaks in Asia, notably in Taiwan in 1998 where an estimated 1.3 million people were infected resulting in the death of 78, of which 71 were children\(^{160}\). From 2008 until 2012, more than 7 million cases have been reported in China with more than 267,000 confirmed cases and over 2,450 deaths\(^{15}\). EV71 is now responsible for seasonal outbreaks of HFMD that occur around May and October each year\(^{15,153}\).

1.3.2 Enterovirus 71

1.3.2.1 Genome, proteome and structure

The virus is non-enveloped, and composed of a 30 nm-wide icosahedral capsid that encompasses the viral genome. The capsid is made up of 60 protomers, each composed of
4 proteins: VP1, VP2, VP3 and VP4, the latter being located on the internal side of the virion (Figure 1.8). The EV71 genome is composed of a single strand of positive-sense RNA that possesses a covalently-linked VPg protein on its 5’ end, followed by an internal ribosome entry site (IRES), the coding region, and a 3’ untranslated region followed by a poly-A tail. The genome acts as a mRNA that encodes for a single polyprotein, which is subsequently cleaved into 3 precursor proteins P1, P2 and P3 by viral proteases, and finally into 11 distinct viral proteins\textsuperscript{153,156}. As shown in Figure 1.8, these proteins are the 4 structural proteins VP1–4, the proteases 2A and 3C, the viroporin 2B, the chaperone 2C, the replication complex-associated protein 3A, the genome-linked protein 3B (or VPg), and the RdRp 3D\textsuperscript{161}.

![Figure 1.8: Structure and genome organisation of EV71. Adapted from Solomon et al.\textsuperscript{156}.](image)

### 1.3.2.2 Genetic diversity

Based on the genetic sequence of the VP1 and VP4 protein, EV71 is divided into 6 genogroups, A to F, although only a few isolates have been reported for the genogroups D to F\textsuperscript{162–164}. The group A comprises the reference EV71 strain BrCr, isolated in California in 1970, while groups B and C, each divided into the subgenotypes B0–B5 and C1–C5, are largely prevalent around the world\textsuperscript{162,165}. The genogroup C is the most common in East and South-East Asia, and contains most of the prevalent EV71 strains. In China, Vietnam, Taiwan, mainly strains of the C4 subgenotype are in circulation. These countries are also
among the most affected by HFMD\textsuperscript{15,156,162,166}.

### 1.3.2.3 EV71 replication cycle

The virus infects cells of the respiratory or gastrointestinal tract by transmission through respiratory droplets or the fecal-oral route, respectively. It then propagates by the haematogenous route where it develops a mild-viraemia. EV71 can sometimes spread to a secondary site of infection such as the CNS, via retrograde axonal transport, that may result in severe neurological infection\textsuperscript{13,153,155}.

Entry into the host cell is achieved via pH- and clathrin-mediated\textsuperscript{167} or caveolin-mediated\textsuperscript{168} endocytosis, that is initiated by binding of the virion to a variety of cellular receptors. The most described EV71 receptors are the human P-selectin glycoprotein ligand-1 (PSGL-1)\textsuperscript{169} and the scavenger receptor B2 (SCARB2)\textsuperscript{170}, although binding to heparan sulfate, annexin II or sialylated glycans has also been reported\textsuperscript{171}. EV71 binds SCARB2 via a canyon located close to the 5-fold axis of the virus capsid, and this binding event mediates clathrin-dependent endocytosis, followed by virus uncoating in the host cell. Binding to PSGL-1, however, occurs only in 20\% of EV71 strains and does not trigger virus uncoating.

Following entry, the viral genome is translated into viral proteins while being replicated in cytoplasmic membrane vesicles. The genome is packaged into newly-synthesised capsids, and the virus exits the cells upon cell lysis (Figure 1.9).
Figure 1.9: Life-cycle of *Picornaviridae*. dsRNA: double-stranded RNA. Adapted from Whitton et al.\textsuperscript{172}. 
1.4 Drug Discovery and Inhibitors of hPIV-3, hRSV and EV71

To date, there is neither vaccine nor drug treatments against hPIV, hRSV and EV71 infections. Only a prophylactic antibody therapy exists to prevent disease caused by hRSV, although it is not widely available. This section will review the methodologies and principles of drug discovery research, as applied to antivirals, along with the current progress in anti-hPIV-3, hRSV and EV71 small-molecule inhibitor development.

1.4.1 Introduction to drug discovery

Drug discovery is a process driven by an unmet need for a therapy to a clinical condition. It involves the identification of compounds having an effect, agonistic or antagonistic, towards a biological system or molecular target. Compounds identified by this process, referred to as hits, are subsequently refined to enhance their potency to their target, and become lead compounds. The lead compounds are in turn optimised during a step of drug development before they can be approved for pre-clinical studies and clinical trials. In the course of this phase, lead compounds are further evaluated for their efficacy and safety and developed to maximise their chance to obtain approval by regulatory authorities. It is estimated that it takes 10–17 years for a drug candidate to reach the market from hit discovery, noting that a candidate takes about 5 years to reach lead optimisation, and that the overall associated cost often exceeding 1 billion US dollars.\(^\text{173,174}\)

When a drug discovery project is initiated, either a molecular target has been identified and the discovery will be target-based (TBD), or the molecular target is unknown and the discovery will be phenotypic (PBD).\(^\text{175}\) Phenotype-based drug discovery is empirical in early stages, with an aim to select compounds through a screening process that have a biological effect towards a system, without prior knowledge of their target or of the molecular mechanism underlying the effect observed. Target-based drug discovery, on the other hand, is a rational approach aiming to design or select compounds having an effect towards a specific molecular target. Between 1999 and 2008, out of 75 new molecular entities (NME) with novel mechanisms of action approved by the FDA 37.3% were from PBD and 22.7% were from TBD. In comparison, out of 164 NME with known mechanisms of action (or follower drugs) 18.3% were from PBD and 50.7 were from TBD.\(^\text{176}\)
As described in Sections 1.4.1.2 (p. 32) and 1.4.1.3 (p. 34), and as depicted in Figure 1.10, these two methods are different ways of identifying hits that can follow one another in later stages of a drug discovery project.

Figure 1.10: Target and phenotype based approaches of drug discovery. HTS: High Throughput Screening. Adapted from Andreux et al.177.

1.4.1.1 General concepts

This brief Section introduces some concepts key to drug discovery, such as the methods by which the potency and toxicity of a compound is evaluated in vitro, as well as the methods allowing to determine at which step of infection an antiviral molecule is active.

1.4.1.1.1 Potency and toxicity The potency and toxicity of a compound in vitro are evaluated in dose-response experiments. They are represented by the IC\textsubscript{50} and CC\textsubscript{50} values, that are concentrations of a compound required to achieve 50% inhibition or cytotoxicity in vitro, and are comparable between compounds tested in similar conditions\textsuperscript{178}. It is also common practice to determine the IC\textsubscript{90} and IC\textsubscript{95} values, as they give a reasonable idea of the minimum dose required to reach the maximum effect.

In vitro potencies of compounds in virology can be measured in a number of ways. In most cases virologists make use of plaque-reduction assays, in which the virus is immobilised by viscous, colloidal or agarose-containing mediums\textsuperscript{179–181}. The virus growth leads to the localised death of cells at the site of primary infection, which leads to the formation of plaques. The number of plaques as well as their size can be measured as means to evaluate the potency of an antiviral. In cases where the virus does not lead to cell death, or when the time to cell-death is too long (over 5 days), infected cells can be
stained after 2 to 3 days with antibodies specific to viral proteins, to reveal foci at the sites of infection. Such methods are called focus-reduction assays\textsuperscript{180,181}. Cell-based enzyme-linked immunosorbent assays (ELISA) are similar to focus reduction assays, with the difference that no immobilisation medium is used\textsuperscript{182,183}. Therefore, there is no need to count or measure plaques, and infection is assessed by colorimetric assays using virus-specific antibodies. Other non-immobilised methods involve titrating the load of released virus from infection media by standard plaque assay, or by quantitative real-time polymerase chain reaction (qPCR)\textsuperscript{184}.

Cytotoxicity is evaluated by measuring the fitness of cells in presence of compound, more specifically their mitochondrial activity. The most prevalent methods assess the amount of adenosine triphosphate (ATP) in cells (ATP-based assays), or the amount of mitochondrial reductases (MTT-based assay). In both cases, the presence of ATP or mitochondrial reductase in cells drives the conversion of colorimetric substrates into products of a different colour, that are quantifiable by absorbance readings\textsuperscript{185,186}.

The selectivity index SI is another indicator of the druggability of a compound, where 

\[ SI = \frac{CC_{50}}{IC_{50}} \]

Thus, the higher the SI value, the lower the compound toxicity and the higher its inhibitory effect. Compounds of high SI have therefore better therapeutic potential\textsuperscript{178,187}.

\subsection*{1.4.1.1.2 Time-of-addition and mode of action}

Early-on in a study where the mode of action of antiviral hit is unknown, it is important to determine at which step of the replication cycle it is most active. It gives valuable information on the potential target, as well as on the mechanism of inhibition. In the context of a viral infection, it can be easily achieved by testing the inhibitor at different stages of infection, in what is called a time-of-addition assay\textsuperscript{188,189}. In preliminary experiments, compounds are tested during virus adsorption (binding) only, after adsorption only, or during all stages of infection. Compounds of higher potency during adsorption are more likely to inhibit binding or entry of the virus, while compounds most active post-adsorption can inhibit any post-entry event of infection (genome replication, protein production, viral assembly or release). To time with greater precision the action of the inhibitor, cells are infected synchronously during a single round of viral replication, about 20 h for hPIV-3 and hRSV for instance, and are
treated with a single dose of compound every 2 h (one infection per time point). The infection is stopped at around the time of virus release, and the viral loads in the infection medium are titrated and plotted on a graph as a function of time. The time of inhibitor addition at which the viral load rises indicates the loss of inhibitor activity. Thus, if the viral load rises early the inhibitor blocks entry, if it rises late it blocks assembly or release, and if it rises in between it is likely to block genome replication or protein production.

### 1.4.1.2 Phenotype-based discovery

PBD serves two purposes: the discovery of compounds of novel activity towards a system, for instance antivirals, and the discovery of novel targets against the system. For that reason, PBD is used either when no druggable targets are available, or when inhibitors of known targets have not led to satisfactory therapies.

#### 1.4.1.2.1 Phenotypic screening

In PBD, novel antivirals are identified by screening in cell-based assays *in vitro*, or less commonly in *in vivo* assays. This is referred to as phenotypic screening and changes in the system’s phenotype, for example changes in the behaviour or biology of a cell, is expected upon treatment\(^ {190} \). In the case of antivirals, a reduction of virus-induced cytopathic effects and viral load is therefore monitored. Thus, a possible advantage of phenotypic screening over target-based screening is that hits are biologically active, and toxicity can be evaluated straight away.

High-throughput screening (HTS) is usually the method of choice for PBD, in which large compound libraries (10,000–100,000 compounds and above) can be tested for hit identification in a fully roboticized fashion. While this method is very efficient, it requires dedicated facilities\(^ {191} \). Semi-HTS, on the other hand, is suitable for smaller libraries of up to several thousands compounds that can be tested partially or solely by hand.

#### 1.4.1.2.2 Target deconvolution

Molecules identified by TBD can not be readily optimised unless a molecular target has been characterised. Even functional screenings, specific to cellular functions or metabolic pathways, are not sufficient in themselves to give structural insight on the modifications needed to be done to improve the potency of a compound if the target is unknown. In that case, molecular derivatives and structural
analouges can be synthesised to refine hits, although the design strategy will be more empirical rather than structure-based. By knowing the target, a lot of information emerges regarding the function and toxicity of compounds, and the extent of their biological activity. For these reasons, it is imperative to understand the molecular mechanisms of novel drug candidates. Target deconvolution is a complex task of characterising the biological target of hits identified by phenotypic screening. Several methods can be employed to achieve this goal and the end result, in the best of cases, is a proof of direct interaction between the compound and the biological target.

**Chemical proteomics** allows the direct identification of drug targets from a biological medium. In such methods, drugs or small molecules are linked to a solid surface or resin and are incubated with the biological sample, such as a cell lysate. After elimination of non-specific interactions by extensive washing, the linked compound has the ability to retain any partner or protein bound to it with sufficient affinity. The compound is then eluted from the surface or resin along with any binding partner, and the nature of the bound proteins is determined by mass spectrometry. While these methods are efficient in identifying binding partners, they are rather time consuming. In addition, it is hard to depict interactions of weak affinity, or to discriminate partners that bind directly versus indirectly to the compound. Lastly, the linker can pose an issue as it can prevent a good presentation of the molecule to its target.

**Resistance selection** is another method used to single out molecular partners involved with an inhibitor effect. It makes use of the high mutation rate of RNA viruses and the natural occurrence of quasi-species to specifically select populations resistant to an antiviral agent. To do so, pathogens are serially passaged in presence of antiviral to ensure that sufficient cytopathic effect (CPE) can be observed at each passage. The concentration of compound, that results in approximately 50% blockade of virus-induced CPE at the beginning, is increased at each passage until a high non-toxic concentration has been reached. The resulting species is then amplified and sequenced, to be compared to a reference strain. Any virus obtained through this process will have been selected by drug resistance, and will possess one or multiple escape mutations associated with
its resistant phenotype. The mutations responsible for the resistance do not necessarily indicate the molecular target of the antiviral. Compounds identified by phenotypic screening can target the virus as well as the host cell, thus this method is a powerful way of discovering viral protein partners involved with inhibition by the compound, that are important enough in the virion fitness to allow the selection of resistant mutants. These partners can be viral interactors of cellular proteins, as well as direct partners of the antiviral itself.

**Other methods of target deconvolution** involve the modulation or knock-down of cellular pathways with the use of chemical tools or antibodies, and short-interfering RNAs (siRNA) respectively. Such pathways can also be upregulated using chemical agonists or genetic engineering. Additionally, phage display or yeast-three-hybrid are powerful methods to identify binding partners of biomolecules, through the expression of cDNA libraries coding for multiple proteins\textsuperscript{193}. However, small-molecules usually require to be tagged or linked and are therefore not used in their native conformation.

It is only after a target has been identified that target-based discovery takes over phenotype-based discovery, so long as a specific effect of the hit on the target can be detected using functional screenings at the macromolecular level, or as a 3-dimensional (3D) structure of the target is available.

### 1.4.1.3 Target-based discovery

It has been emphasised in the previous section that phenotype-based discovery is an empirical approach. On the contrary, target-based discovery (TBD) follows rational methodologies to identify modulators of specific molecular targets. TBD has been successfully used to initiate a drug discovery project\textsuperscript{197}, or to follow-up on a PBD project after target deconvolution\textsuperscript{198,199}. As a prerequisite to TBD, a drug target must have been characterised and must be essential in disease progression.

#### 1.4.1.3.1 Overview

The hit identification in target-based discovery can be done in several ways. Small molecules can be rationally designed and synthesised to specifically in-
hibit a molecular target, or libraries can be screened against the target. In either case, compounds are primarily tested biochemically (for binding with the target or inhibition of enzymatic activity for instance) on recombinant proteins, cells expressing the target of interest, or even virus particles carrying enzymes. Hits are then tested in a biological assay, such as *in vitro* infection, to validate their therapeutic capability. Because hits from a biochemical assay do not necessarily have the right pharmacological properties to be validated in cell-based assays (unlike hits from PBD), they need to be optimised using drug design/chemical synthesis and re-tested until a lead can be found. For targets that have a 3-dimensional structure available, screening by *in silico* docking can be performed for hit identification. Other *in silico* analysis can also be performed for in-depth characterisation of hit-target interactions, and greatly contribute to the improvement of lead compound design.

### 1.4.1.3.2 Screening

A primary screening in TBD allows the selection hits according to their ability to alter the function of a protein, protein-ligand interactions or protein-protein interactions. In this case, “screening” refers to the primary biochemical assessment of compounds, whether they are isolated molecules or from a compound library. They are typically evaluated against enzymatic functions, or on their protein binding affinity using interaction analysis methods such as surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), NMR, or enzyme kinetics. Hits can therefore be discriminated in one of three ways:

1. by testing compounds specifically designed to bind a molecular target
2. by HTS of compound libraries
3. by screening compounds using *in silico* simulations

The first method relates to rational drug design, where inhibitors are designed and synthesised taking into consideration the molecular structure of the target receptor. Such molecules are either based on the natural ligand of a receptor, or designed to bind a specific region or binding pocket of the protein of interest. The influenza virus neuraminidase inhibitor zanamivir (Relenza®, GlaxoSmithKline) is the first commercialised antiviral drug originating from rational drug design, and still is one of the best examples of this strategy to-date. It is a transition state-like analogue based on a sialic acid...
framework, similar to the natural substrate of neuraminidases, and was designed using the crystal structure of influenza A virus N2 protein. The transition state is a high energy state in an enzymatic reaction where the structure of a ligand would lie somewhere in between the structure of the substrate and the structure of the product\textsuperscript{207}. Transition state analogues bind very tightly to the enzyme, and make very potent enzyme inhibitors.

The second method relates to high-throughput screening of chemical libraries to segregate enzyme inhibitors or receptor binders. Here again, several strategies can be employed, and depend upon the type of library tested. Organic compound libraries, whether they are approved drugs or natural compound libraries, are used to identify novel inhibitors of enzymes that are not necessarily related to their natural substrate\textsuperscript{201}. Libraries of structural analogues of a given compound, on the other hand, are used to identify better versions of the same compound\textsuperscript{208}. A third strategy, referred to as fragment-based screening, aims to screen low-molecular weight fragments (<300 Da – about the weight of Neu5Ac) to build up potent inhibitors\textsuperscript{209}. The idea is to combine several fragments of low affinity (sub-millimolar) to create larger compounds with increased potency. These fragments, by virtue of their small size and low steric environment, are more likely to fit regions of a protein otherwise not very accessible by naturally-occurring larger molecules.

Lastly, \textit{in silico} docking simulations are used to predict the likelihood of a molecule to bind a receptor of interest\textsuperscript{210}. A 3D structure of the receptor solved by NMR spectroscopy, X-ray crystallography, or homology modelling must be available. Molecular docking makes use of force fields to simulate the binding of a large number of spacial conformations of a ligand in a defined region of a protein, such as an active site. Essentially, it evaluates the free energy of binding of the ligand by calculating various parameters depending on the program used. In the case of Autodock 4 for instance\textsuperscript{211}:

\[
\Delta G_{\text{binding}} = \Delta G_{\text{vdW}} + \Delta G_{\text{elec}} + \Delta G_{\text{H-bond}} + \Delta G_{\text{desolv}} + \Delta G_{\text{tor}}
\]

where \(\Delta G_{\text{binding}}\) is the free energy of binding, and is comprised of the free energies of van der Waals contacts, electrostatics, hydrogen-bonding, desolvation and torsion that are represented by \(\Delta G_{\text{vdW}}, \Delta G_{\text{elec}}, \Delta G_{\text{H-bond}}, \Delta G_{\text{desolv}}\) and \(\Delta G_{\text{tor}}\) respectively.
It is important to note that *in silico* docking simulations only predict potential binding affinities, and results obtained through these methods need to be corroborated by biochemical or cell-based approaches.

### 1.4.1.3.3 Hit-to-lead phase

Hits obtained by rational drug discovery or drug design need optimisation and refinement to become leads. As the capacity of hits they have been validated in biochemical assays and have satisfied a certain threshold, but it does not mean that they have good activity in cell-based assays. They may have poor activity in cell-based assays, or simply not have a good enough affinity for their target. As a consequence, their chemical structure must be modified to enhance their hydrophilicity, flexibility, or polarity for instance, so that they can be better accommodated by their receptors. This link between the structure of compounds and their activity is referred to as structure activity relationship (SAR), and is a driving force in rational drug design\textsuperscript{212}.

Reasoned hit refinement implies that the binding mechanism of the inhibitor must be well described, in order for the functional groups involved to be rationally oriented or adjusted. Several methods can therefore be employed for prediction of binding mechanisms, mapping of binding epitopes, characterisation of binding affinity and thermodynamics, or determination of the mode of inhibition of enzyme inhibitors.

*In silico simulations* are used in hit refinement processes to predict the binding mechanism of a small molecule to its receptor (molecular docking), and to predict the receptor structural changes induced by binding of the inhibitor or the stability of a receptor-ligand interaction\textsuperscript{126,213}. These predictions are typically performed by molecular dynamics (MD) experiments, which aim to simulate the motions and trajectories of atoms in a 3D environment using defined force fields, and taking into consideration the ionic strength of the medium, water molecules, temperature, and all possible interactions with nearby atoms or molecules\textsuperscript{214}. MD experiments are typically simulated over very short periods of time, in the order of the nanoseconds. In a more general approach, computational methods are key to predicting what functional groups of an inhibitor could be modified to enhance its affinity towards its receptor, by analysing its relative position within the molecular target.
Binding epitope mapping is a method used to determine which atoms of a small molecule are engaged in binding with a molecular receptor. While in silico methods are useful to predict such events, they are only valid as long as other biophysical methods can support the observations. The most powerful way of characterising receptor-ligand interactions is to obtain 3D coordinates of the complex using X-ray crystallography or solution-based NMR spectroscopy. Other methods exist that allow one to map the binding epitope of small molecules to receptors with precision such as saturation transfer difference NMR spectroscopy (STD-NMR) or fragment-based screening.

STD-NMR spectroscopy is an NMR technique that enables the identification and characterisation of ligand protons that interact with a receptor at an atomic level. It is based on nuclear Overhauser effects (NOE), and on the fact that biomolecular interactions are reversible processes. NOEs reflect the transfer of spin polarisation states of nuclei from protons that are spatially close, in a dipolar interaction. In STD-NMR the receptor, protein or whole virus for instance, is selectively saturated at a given frequency, resulting in the acquisition of an “on-resonance” spectrum where the resonance signals of the receptor are absent. During this event, transfers of saturation by NOE occur between the protons of the receptor and the protons of the ligand bound to the receptor. In transient interactions, the un-bound ligand will retain this saturation, and the resonance signals of the ligand will be affected as observed in the spectrum. Another spectrum is also acquired without saturation of the receptor, the “off-resonance” spectrum, where the resonance signals of all ligands present in the sample are observed. The STD spectrum is created by subtracting the “on-resonance” acquisitions from the “off-resonance”, where only the ligand protons involved in interaction with the receptor are observed. The major benefit of this method is that the ligand protons that are in close contact with the receptor will have stronger STD-NMR signals. It is therefore possible, by analysis of the relative intensity of proton signals, to map the binding epitope of a ligand to its receptor. Furthermore, experiments can be performed with whole viruses; the major advantage being that on the one hand the virus can be selectively saturated without harm to the ligand, and on the other hand its large size yields longer correlation times (rotational motions) and thus more efficient saturation transfers.
Fragment-based approaches can be used to discover novel hits, as discussed in Section 1.4.1.3.2 (p. 35), and they can also serve to determine what part of a compound binds to its target. In this case, fragments of large molecules are generated, and each of the fragments are tested for binding in NMR experiments. By analysing binding affinities for each of them, it is possible to reconstitute the binding epitope of the whole ligand\textsuperscript{218}.

**Binding kinetics, affinity and thermodynamics** are important properties of receptor-ligand interactions, since they determine whether an interaction is weak, strong, transient, stable or energetically favourable. The equilibrium dissociation constant $K_D$ (unit: mol) is a common measure of the affinity of a ligand for its receptor. Indeed, it represents the ratio of the dissociation rate constant of the ligand, $k_{off}$ (unit: s$^{-1}$), to its association rate constant $k_{on}$ (unit: M$^{-1}$s$^{-1}$). The smaller the $K_D$, the higher the affinity of the ligand. Thus:

\[
A + B \xrightleftharpoons[k_{on}]{k_{off}} AB \quad \text{and} \quad K_D = \frac{k_{off}}{k_{on}} = \frac{[A][B]}{[AB]}
\]

where [A] and [B] are the individual concentrations of molecules A and B, and [AB] is the concentration of the complex AB. $K_D$ is linked to the Gibbs free energy change in standard conditions at equilibrium $\Delta G^\circ$ through the relation:

\[
\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = RT \ln K_D
\]

where $\Delta G^\circ$ relates to the spontaneity of the reaction, $\Delta H^\circ$ is the enthalpy change in the system, or the amount of heat absorbed or released during a reaction, $T$ is the temperature of the system in K, $\Delta S^\circ$ is the variation of entropy in the system, or the degree of order of the system, and $R$ is the ideal gas constant. In favourable conditions, $\Delta G^\circ < 0$, $\Delta H^\circ < 0$, $\Delta S^\circ > 0$.

The binding affinity of a ligand can be measured by STD-NMR, but the methods of choice remain surface plasmon resonance (SPR) or isothermal titration calorimetry (ITC)\textsuperscript{206}. SPR relies on the change of mass at the surface of a gold-plated chip, that is evaluated in real-time by measuring the change of the reflection angle of polarised light going through a prism and illuminating the surface. It measures kinetics and dissoc-
tion equilibrium constants, from which the Gibbs free energy of the system can be calculated\textsuperscript{219}. ITC, on the other hand, relies on direct thermodynamic analysis by measuring a variation of temperature between 2 cells. The first one, the sample cell, is in contact with the biological sample while the other one, the reference cell, is not. When an interactant is put in contact with the sample, a variation of temperature is detected in the sample cell. The amount of energy needed for the temperature of reference cells to match the one of the sample cell is measured, and from it the dissociation equilibrium constant, as well as Gibbs free energy, entropy and enthalpy changes can be determined. In addition, the stoichiometry of the reaction can be calculated (number of ligand binding sites on the receptor)\textsuperscript{220}.

**Enzyme inhibitors** alter the catalytic or anabolic function of enzymes by modifying the way they react with their substrate. Different types of inhibitors may exhibit different types and levels of enzyme inhibition depending on their mode of action\textsuperscript{221}. It is important to determine the mechanism of inhibition in order to understand where the inhibitor binds on its target, and to determine what amino acids maybe involved. Enzyme inhibitors are generally divided into 3 major types of inhibition: competitive, non-competitive, un-competitive. It is possible to determine the type of inhibition of an inhibitor by measuring the initial velocity $v_i$ of the enzyme at increasing concentrations of substrate [S], at several concentrations of inhibitor [I]. The analysis of the resultant Lineweaver-Burk plot, that represents the linear regression of $\frac{1}{v_i} = f(\frac{1}{[S]})$ for each [I], allows the graphical determinations of the various kinetic parameters of the enzyme as shown in Figure 1.11 (p. 41).

A competitive inhibitor is often structurally related to the substrate, and binds in its place so both of them can not bind the enzyme simultaneously. The maximum rate of the reaction will therefore not be affected by the inhibitor as either the one or the other is bound to the enzyme at a given time. An increase in substrate concentration overcomes inhibition as less inhibitor is able to bind the enzyme. As a consequence, the maximum velocity of the \textit{apo}-enzyme $V_{\text{max}}$ and the apparent maximum velocity of the enzyme in presence of inhibitor $V_{\text{max}}^{\text{app}}$ are equal ($V_{\text{max}} = V_{\text{max}}^{\text{app}}$), while the constant of Michaelis-Menten $K_M$, defined as the concentration of substrate needed to achieve 50%
of \( \text{V}_{\text{max}} \), is increased in the presence of inhibitor \( (\text{K}_M < \text{K}^\text{app}_M) \).

A non-competitive inhibitor, on the other hand, affects the enzyme by binding to a site distinct from that of the substrate binding site, either in the apo-form of the enzyme or in the enzyme–substrate complex. Thus, inhibition cannot be abolished by increasing the concentration of substrate, and \( \text{V}^\text{app}_{\text{max}} \) varies as a result of inhibitor binding but \( \text{K}_M \) remains constant. In other words, \( \text{V}_{\text{max}} > \text{V}^\text{app}_{\text{max}} \) and \( \text{K}_M = \text{K}^\text{app}_M \).

Lastly, in an un-competitive inhibition the inhibitor only binds the enzyme–substrate complex, meaning that substrate binding induces an enzyme conformational change required for the inhibitor to bind. In this case, both \( \text{V}_{\text{max}} \) and \( \text{K}_M \) are reduced in the presence of inhibitor, \( \text{V}_{\text{max}} > \text{V}^\text{app}_{\text{max}} \) and \( \text{K}_M > \text{K}^\text{app}_M \).

![Lineweaver-Burk plots](image)

Figure 1.11: Major types of enzyme inhibition as represented by Lineweaver-Burk plots. The x-intercept in presence of a competitive or un-competitive inhibitor is \(-1/\text{K}^\text{app}_M\), while the y-intercept in presence of a non-competitive or un-competitive inhibitor is \(1/\text{V}^\text{app}_{\text{max}}\).

From the kinetic parameters of an enzymatic reaction, the inhibition constant \( \text{K}_i \) of the inhibitor can be determined. It corresponds to the dissociation constant of the enzyme-inhibitor complex, and as such is an equivalent of the previously mentioned \( \text{K}_D \) value, where \( \text{K}_i = \frac{[E][I]}{[EI]} \) in the case of a competitive inhibitor, and \( \text{K}_i = \frac{[ES][I]}{[EST]} \) in the case of a non-competitive inhibitor. The \( \text{K}_i \) can be calculated from the determined \( \text{V}^\text{app}_{\text{max}} \) and \( \text{K}^\text{app}_M \) values at a given [I]:

- For a competitive inhibitor: \( \text{K}^\text{app}_M = \text{K}_M(1 + \frac{[I]}{\text{K}_i}) \)
- For a non-competitive inhibitor: \( \text{V}^\text{app}_{\text{max}} = \frac{\text{V}_{\text{max}}}{1 + \frac{[I]}{\text{K}_i}} \)
- For an uncompetitive inhibitor: \( \text{V}^\text{app}_{\text{max}} = \frac{\text{V}_{\text{max}}}{1 + \frac{[I]}{\text{K}_i}} \) and \( \text{K}^\text{app}_M = \frac{\text{K}_M}{1 + \frac{[I]}{\text{K}_i}} \)
Similarly with $K_D$ values, the lower the $K_I$ value the higher the affinity of the inhibitor for the enzyme.

The sections above provide guiding principles in the rational design of novel inhibitors. As discussed, drug discovery is iterative in the sense that new compounds need to be synthesised, evaluated and modified back and forth until a suitable lead is found. They must possess drug-like properties, and undergo optimisation, pre-clinical and clinical evaluation before being approved for market. Unfortunately, many drug candidates fail in early and later stages of clinical trials.

### 1.4.2 Drug repositioning

Early drug discovery as presented in Section 1.4.1 (p. 29) can take 3-6 years to complete from the first screening or target identification until an optimised lead is obtained. Pre-clinical studies, involving in vivo evaluation of lead compounds in animal models, pharmacokinetics (PK) and pharmacodynamics (PD) studies, take on average another 2 years to complete. Finally, it takes about 6 years to achieve phase I (safety), II (efficacy) and III (efficacy in large cohorts) of clinical trials and another 3 years for FDA final evaluation and post-approval phase IV monitoring.

There is a significant risk of failure from each of the 3 principal phases of clinical trials, principally due to a lack of efficacy or safety in phase II and III\(^{222,223}\) (Figure 1.12A, p. 43). From 2003 to 2011, drug candidates entering phase I clinical trial had a 15.3% chance only of being approved for their main indication after application to the FDA, against 10.3% for all of their indications\(^{223}\). In addition, as shown in Figure 1.12B (p. 43), drug candidates mainly failed in validating phase II clinical trials since only 39% of the candidates went through to phase III trials for their main indication, and 32% for all of their indications (in cases where a candidate was applied for more than one indication). However, all is not lost for drug candidates that have failed clinical trials as they can re-enter a previous phase for an indication different from their original one. This is also the case for approved drugs, that are being more and more repositioned (or repurposed) for new indications\(^{174,224,225}\).
Two of the most well-known examples of successfully repurposed drugs are sildenafil (Viagra®, Pfizer) and thalidomide. Sildenafil was originally intended to treat a heart condition, angina, in the 1980s. It failed clinical trials due to poor PK for angina, while patients reported strong erections as a side-effect. It was then re-introduced in clinical trials for the specific treatment of erectile dysfunction\textsuperscript{226,227}. Thalidomide, on the other hand, was sold in the 1950s as a treatment for morning sickness during pregnancy. Shortly after its introduction to market, it was found to induce severe birth defects to newborns with 10,000–15,000 reported cases and was withdrawn. Since then thalidomide has been prescribed for specific cases, including tuberculosis, the treatment of erythema nodosum lapposum (a complication of leprosy), and multiple myeloma (in combination with dexamethasone)\textsuperscript{225,226}.

A few recent studies suggest that approved drugs could show promise as antiviral agents. Lejal and colleagues have discovered the anti-inflammatory drug naproxen as an inhibitor of influenza A virus nucleoprotein by \textit{in silico} docking and molecular dynamics\textsuperscript{228}, while Clouser and colleagues have reported that a combination of decitabine (Dacogen®, Otsuka) and gemcitabine (Gemzar®, Eli Lilly), originally prescribed for myelodysplastic syndrome and various cancers respectively, could be used against HIV infections\textsuperscript{229}.

Figure 1.12: Causes of failure and chances of success in clinical trials. A. Main causes of failure in phase II and III from 2007 to 2010. B. Likelihood of success (%) for a drug candidate’s lead and all indications in each of the 3 main phases of clinical trials, until new drug application (NDA) or biologic licence application (BLA) to the FDA from 2003 to 2011. Adapted from Ledford et al. and Hay et al.\textsuperscript{222,223}.
1.4.2.1 Pros and cons of drug repositioning

Repositioning drugs brings many advantages over classical drug development. As described by Padhy & Gupta\textsuperscript{173}, six types of drugs or drug candidates can be repositioned, the 6\textsuperscript{th} is the most difficult to bring to a new market:

1. A drug candidate being developed for several indications
2. A drug candidate that has failed phase II or III but has good safety
3. A drug that was discontinued
4. A generic drug or a drug whose patent has expired
5. A drug marketed in a different part of the world
6. A drug marketed worldwide

All of these drugs have been advanced to at least phase II clinical trials and have therefore well-established pharmacological properties (PK/PD) and safety profiles. As a consequence, they do not necessarily need to undergo phase I in case of repurposing. Naturally, most of the steps in early drug development can also be avoided, since the candidates are optimised molecules that possess drug-like properties. Because they can be advanced quickly to later phases of drug development, the time-frame to reach the market is often shorter for a repositioned drug, and the associated cost is lower compared to classical strategies. Therefore, the risk exposure to failure for pharmaceutical companies is also reduced. The time-to-approval is estimated to be 3–12 years for repurposed drugs, against 10–17 years for \textit{de novo} drugs\textsuperscript{174} (Figure 1.13, p. 45).

The principal obstacle in drug repositioning projects is the licensing of the end product\textsuperscript{174,230}. As introduced above, a repurposable drug can be off-licence or a generic drug, or can be marketed under a pending regional or worldwide license. The latter (type-6) is the most difficult to repurpose, as it is protected in all countries for its method of use (MAU) and composition of matter (COM). To obtain a new licence for another indication, 3 options can be considered: working around the MAU if the drug was not originally protected for many applications, changing the mode of administration of the drug (oral to intravenous for instance), or changing the COM if the drug works in combination with another drug. For a drug under a regional licence, one may consider to re-licence for another part of the world.
1.4.2.2 Methods for drug repositioning

There are over 7700 drugs listed on the DrugBank database, of which 6000 are experimental, over 1700 are approved drugs, and more than 1500 are FDA-approved small-molecules\textsuperscript{231–234}. All these molecules are a valuable tool-box for drug repurposing, considering that a lot of information about their mode of action, biological target, or interaction with other molecules is available in literature and various databases. They can be used in two ways: via activity-based methods, or computational methods\textsuperscript{224,225,235}.

Most of the approved drugs can be purchased in the form of drug libraries, that can be screened in phenotype-based or target-based projects \textit{in vitro} or \textit{in vivo}. These activity-based methods are powerful, since they allow the direct evaluation of the drugs for new indications. In addition, when drugs show activity in phenotype-based assays, they can also serve as tools for fundamental research. Because their mechanism of action is often known, it is possible to establish links between their target-pathway and the disease or pathogen of interest.

The other way of identifying repurposable drugs is by the use of computational tools and data-mining to predict drug-target relationships and potential new uses for existing drugs\textsuperscript{224,236}. While such approaches are time consuming, they are significantly cheaper than activity-based approaches. They rely on the knowledge acquired during basic research that led to the drug and during drug development, and take into account activity,
interaction, PK/PD and toxicity data. Finally, libraries can also be screened in silico in cases where the structure of the receptor of interest is known, to pave the way to subsequent activity-based investigations.

1.4.3 Small-molecule inhibitors of hPIV-3, hRSV, and EV71 infection

As previously described, the paramyxoviruses hRSV and hPIV are serious threats to infants, the elderly and immunocompromised. The overall economic burden associated with paediatric infections alone caused by these viruses is estimated to be over $US600 million per year in the US\textsuperscript{23,29,45,237}. EV71, on the other hand, is the cause of several hundred HFMD-associated deaths among the paediatric population each year in China alone, while up to 50% of HFMD patients require hospitalisation\textsuperscript{15,238}. Unfortunately, there are neither drugs nor vaccines to effectively treat or prevent infection from either of these pathogens.

1.4.3.1 Inhibitors of hPIV-3 and hRSV infection

An immuno-prophylactic treatment does exists to prevent hRSV infections, the humanized murine monoclonal anti-hRSV F antibody palivizumab (Synagis\textsuperscript{®}, MedImmune)\textsuperscript{239}. It is recommended as a prophylactic treatment only in severe cases associated with premature births, immunocompromised children, and children suffering with congenital heart disease or chronic lung disease. While palivizumab seems to lower the burden of hRSV infection in these selected populations, its cost-effectiveness remains questionable\textsuperscript{237,240–243}. It is a highly expensive treatment (>US10,000/season/infant\textsuperscript{244}), that can not be reasonably used to prevent infection on a worldwide scale. Several other monoclonal antibodies targeting F and G are being developed for the treatment of at-risk patients, however they will most likely face the cost-effectiveness issue of palivizumab\textsuperscript{245–247}.

A lot of effort is currently being made towards the discovery of antivirals and vaccines against hRSV and hPIV-3 infections. Among the most promising vaccines are hPIV3cp45, a cold passage-attenuated hPIV-3 currently undergoing phase II clinical trials for hPIV-3 infections\textsuperscript{248}, and MEDI-534 (rB/hPIV3-RSVF), a chimeric bovine/human hPIV-3 and hRSV virus (bovine hPIV backbone with hPIV-3 HN, F, and hRSV F) undergoing phase I
clinical trials for hPIV-3 and hRSV infections\textsuperscript{249,250}. As described in the following sections, good progress has been made towards the discovery of anti-hPIV-3 and -hRSV small molecules, with the number of drug-candidates being greater against hRSV infection.

1.4.3.1.1 hPIV-3 hPIV-3 possesses a haemagglutinin-neuraminidase, and as described in previous sections is responsible for cellular receptor recognition and cleavage, as well as stabilisation and activation of viral fusion. Therefore, most of hPIV-3-targeted antiviral research has been focussed on inhibitors of HN, following the strategy that was adopted for influenza virus\textsuperscript{197}. The structures of the principal HN inhibitors can be found on Figure 1.14, p. 48.

Zanamivir is a sialic acid derivative and has been tested on the hPIV-3 neuraminidase activity, haemagglutination and replication \textit{in vitro} and has been initially shown to inhibit the neuraminidase function and viral propagation \textit{in vitro} with a potency of 250 µM and 25 µM, respectively\textsuperscript{251}. However, it was later shown that zanamivir acted on infection by blocking receptor recognition and fusion\textsuperscript{62}. These results served as a proof of concept for the subsequent development of sialic acid-based inhibitors of HN, from which several unsaturated sialic acid (Neu5Ac2en or DANA) derivatives were synthesized. The crystal structure of NDV HN, which is closely related to hPIV-3 HN, was solved in complex with Neu5Ac2en and suggested that a large cavity around the hydroxyl group at the C-4 position of the ligand could be filled to improve the potency of Neu5Ac2en-based inhibitors\textsuperscript{123,252,253}. Several laboratories followed this strategy to synthesise C-4-substituted compounds designed on the NDV HN structure such as BCX-2798 and BCX-2855 (4-azido and 4-dichloromethanesulfonylamino functionalities respectively)\textsuperscript{254}, or compounds with hydrophobic 4-alkyl substituents\textsuperscript{255}. All of these compounds have mid- to low-µM potency against the hPIV-3 sialidase or infection \textit{in vitro}, but only the BCX compounds have been pursued in animal pre-clinical studies against hPIV-1/3. In these pre-clinical studies, recombinant Sendai viruses that incorporate hPIV-3 F and HN, or hPIV-1 HN to infect 129x1/SvJ mice were used, and the compounds were administered intra-nasally. Although they showed antiviral efficacy in prophylaxis models with lethal doses of virus, the compounds only had efficacy in treatment models with low non-lethal doses of virus\textsuperscript{113,256}. None of these compounds have yet entered clinical trials.
Figure 1.14: Sialic acid-based inhibitors of hPIV-3 HN. 1, Neu5Ac; 2, Neu5Ac2en; 3, zanamvir; 4, BCX-2798; 5, BCX-2855. The positions of the principal carbon atoms are labelled in red on compound 1.

The only other treatment currently under investigation for hPIV infection, although not a small molecule, is the sialidase fusion protein DAS181. While originally designed for the treatment of influenza virus infection\cite{257-259}, it shows promising results in the treatment of severe cases of hPIV infections\cite{260-264}. Phase II clinical trials are soon to commence on immunocompromised patients suffering from ALRI\cite{265}. Finally, the broad-spectrum antiviral ribavirin is not used for hPIV-3 infection, as it fails to show significant efficacy\cite{261,266}.

1.4.3.1.2 hRSV As mentioned in the preamble of this Section, there are more drug-candidates under investigation for the treatment of hRSV infection, since it causes more disease with a higher associated economic burden than its hPIV counterparts. Unlike anti-hPIV compounds that mainly inhibit one protein (HN) there exists inhibitors of several hRSV proteins such as F, G or N. However, most of these compounds are fusion inhibitors. The broad-spectrum antiviral ribavirin is the only licensed drug to treat hRSV infection, but it is toxic and has poor efficacy\cite{267-269}. The following list of inhibitors is not exhaustive, but describes compounds currently in clinical trials or having encouraging antiviral properties.

**F inhibitors** The most potent fusion inhibitor is GS-5806 (Gilead Sciences, Inc.), is a compound based on a pyrazolopyrimidine scaffold that has achieved a phase II clinical trial on RSV-challenged healthy volunteers. \textit{In vitro}, it had a strong potency of 0.4 nM (IC$_{50}$) in cell-based assays against reference strains and clinical isolates. It was shown to block fusion, and strains resistant to the compound acquired mutations within the fusion
peptide and the heptad repeat region 2 of the protein, confirming F as the target\^{270,271}. It has been registered for a phase II clinical trial using hospitalised adults with hRSV infections (identifier NCT02135614).

BMS-433771 was developed by Bristol-Myers Squibb and was the result of a lead optimisation process from a hit that was obtained from a cell-based CPE screen of an in-house library. The compound was shown to block hRSV-induced fusion, and infection in vitro of a variety of standard and clinical strains with potencies in the low nM range\^{196}. hRSV strains possessing mutations in the fusion peptide and in the heptad repeat region 2 of F were resistant to BMS-433771 treatment, confirming F as the compound’s target. The compound reduced viral titres in lungs of BALB/c mice by up to 1.5 logs, while having a lesser efficacy in the cotton rat model. However, it was shown to have poor efficacy when administered in a treatment model (1 h post-virus inoculation)\^{272}. BMS-433771 has not been registered for clinical trials, and was later discontinued.

The compound VP-14637 (ViroPharma, Inc.) is a triphenolic inhibitor of hRSV F. It has an IC\text{50} of 1–5 nM on infection in vitro, and resistant-mutants were shown to acquire mutations on the heptad repeat region 2 as well as in between the two heptad repeat regions\^{273}. In the cotton rat model, the lung viral titre was reduced by up to 2.3 logs at a dose of 63–126 µg/kg/day\^{274}. The development of the drug, renamed MDT-637, is now being undertaken by MicroDose Therapeutx and the drug candidate successfully completed phase I clinical trials (identifiers NCT01355016, NCT01489306, NCT01556607).

Finally, TMC353121 (Johnson and Johnson Pharmaceutical Research and Development) is a drug candidate that was a result of molecular modelling optimisation of a hit obtained by in vitro screening of a library that targeted hRSV fusion\^{199}. This strategy was adopted because of the high retention time of the precursor molecule in the various animal (rat, dog, monkey) lungs. The optimised compound TMC353121 reduced viral titres in the lungs of BALB/c mice up to a log in a treatment model, with much lower retention times\^{198}. The compound has not yet been registered for clinical trials.
Other inhibitors  Two other inhibitors of hRSV infection are worth mentioning. They are MBX-300 (formerly NMSO3) and RSV604, inhibitors of hRSV G and N proteins, respectively. MBX-300 (Microbiotix, Inc.) is a tetra-sulfated sialyllipid with high nM \textit{in vitro} antiviral potency. It reduced the viral load in the lungs of cotton rats by 1.4 and 1.3 logs in treatment and prophylactic models respectively\textsuperscript{275}. While initially suspected to act on hRSV binding to the host cell, the compound was later shown by resistance selection to target hRSV G protein\textsuperscript{276}. No further advances have yet been made public.

The only N protein inhibitor available to date is RSV604. It is a benzodiazepine analogue that was obtained by rational refinement of a hit identified from the screening of a 20,000-compound library on hRSV-infected cells\textsuperscript{182,277}. It had good \textit{in vitro} potency in the high nM range on various reference strains and clinical isolates of hRSV. The generation of RSV604-resistant mutants led to the identification of mutations solely located on the conserved region of the N protein. While no \textit{in vivo} data has been reported, the compound was tested in an HAE model of infection and had a potency of 1 µM\textsuperscript{182}. It has completed phase I clinical trials (identifier NCT00416442), but further developments have not been reported.

Lastly, the siRNA ALN-RSV01 (Alnylam Pharmaceuticals, Inc.) was a promising candidate for the treatment of hRSV infections. The target of this siRNA is the hRSV N protein mRNA, blocking the synthesis of N and therefore infection. It had an IC\textsubscript{50} \textit{in vitro} of 0.7–5 nM, and reduced the viral load in lungs of BALB/c mice by up to 3 logs\textsuperscript{278}. The siRNA has been evaluated in phase II clinical trials with challenged healthy individuals and transplant patients infected with hRSV\textsuperscript{279,280}, but was discontinued. While no further studies on ALN-RSV01 have been reported, a second-generation siRNA, ALN-RSV02, is now being developed\textsuperscript{281}.

1.4.3.2 Inhibitors of EV71 infection

The treatments available against EV71 infection are so far only symptomatic, and not specific to the virus replication. A few compounds, however, have been shown to possess antiviral properties against EV71, both \textit{in vitro} and \textit{in vivo}.

Rupintrivir, or AG7088 (Pfizer), is an experimental drug originally designed for the treatment of rhinovirus infection. It is a nanomolar inhibitor of rhinovirus 3C protease\textsuperscript{282},
that was brought to phase I and II clinical trials. Unfortunately, this compound failed to show significant efficacy in phase II, and its development was later stopped. Since then, rupintrivir has been shown to have nanomolar potency against EV71 infection \textit{in vitro} by also inhibiting the 3C protease, as well as efficacy in the suckling mouse model of infection. It failed, however, to protect 100\% of EV71-infected mice. Given its lack of efficacy against rhinovirus infection, its future as an anti-EV71 drug is uncertain.

Pleconaril (Picovir\textsuperscript{®}, Schering-Plough), an inhibitor of picornavirus attachment and uncoating, was rejected application to the FDA after it showed only limited efficacy in a variety of clinical trials. Based on the compound structure, several derivatives, so-called the WIN compounds, were previously synthesised and demonstrated a high potency against EV71 in the low nanomolar range. They were not further studied. Pleconaril, however, was shown to possess \textit{in vitro} potency and some \textit{in vivo} efficacy against EV71 infection.

Recently, the adenosine analogue NITD008, an inhibitor of flavivirus infection, was shown to inhibit EV71 infection \textit{in vitro} and in a mouse model. NITD008-treated mice were well protected from a virus challenge, and a significant reduction of viral load in a variety of organs was observed.

Finally, ribavirin has antiviral efficacy against EV71 but it is only active at higher dose, thus increasing the risks of toxicity.
1.5 Aims and Outline of the Thesis

Despite the number of small molecules being investigated for the treatment of hPIV, hRSV and EV71 infections, none of them have yet reached the market, let alone entered phase III clinical trials.

So far, there have been no reports of hPIV-3 inhibitors with potencies below the micromolar range. Moreover, the BCX benchmark compounds have only shown prophylactic in vivo efficacy, and can not be considered as good candidates for the treatment of infection. Although there are a few hRSV inhibitors currently in clinical trials, there is still a risk for them to fail during the evaluation process. Efforts towards the discovery of novel anti-hRSV, hPIV-3 and EV71 inhibitors therefore still need to be made, to increase the chances of drug candidates to ever reach the market.

The present study was designed to identify and characterise novel small molecule inhibitors of paediatric viral pathogens using the following multidisciplinary approaches:

1. Target-based discovery of hPIV-3 HN inhibitors, using drug repositioning via target-based screening on the one hand, and rational drug design on the other hand

2. Phenotype-based discovery and characterisation of hRSV infection inhibitors, using phenotypic screening and target deconvolution strategies

3. Phenotype-based discovery and characterisation of EV71 inhibitors using phenotypic screening and drug repositioning strategies

Chapter 1 describes the rationale of the Thesis, and discusses the relevant literature and previous related work. Chapters 2 and 3 reports the discoveries of novel paramyxovirus inhibitors, more specifically of hPIV-3 HN and hRSV respectively. Chapter 4 presents the discovery of a new drug-candidate for the treatment of EV71 infection. Finally, Chapter 5 summarises the results obtained, and addresses future directions.
1.6 References


5. UNAIDS Fact Sheet 2014 2014.


Chapter 1. Introduction


Chapter 1. Introduction


1.6. References


Chapter 2

Target-based discovery of anti-parainfluenza viral agents

2.1 Preface

Parainfluenza viruses are a major cause of respiratory illness in infants and elderly world-wide\(^1\text{-}^4\). Among the four hPIVs, the third serotype is the most concerning as it induces acute lower respiratory distress, sometimes leading to bronchiolitis and pneumonia\(^4\text{-}^5\). The possibilities of prophylaxis or treatment for hPIV-3 infection are very limited, if not non-existent. So far, the global effort in the discovery of anti-parainfluenza drugs, focussed on inhibitors of hPIV-3 HN, has been quite poor with only 2 compounds having made it through animal studies\(^6\text{-}^7\).

This Chapter presents the target-based discovery of novel inhibitors of hPIV-3 HN, that were obtained from two distinct strategies: the fist strategy employed rational design of sialic acid-based compounds to identify high-potency HN inhibitors; the second strategy screened an approved drug library on the sialidase function of HN in to select repositionable drugs.

In 2012, Winger and von Itzstein reported that a loop located in the vicinity of hPIV-3 HN active site, opened in the \textit{apo}-form of the protein, could close upon receptor binding. They suggested that this flexibility could help accommodate ligands larger than sialic acid\(^8\), which could then lock the cavity open.
Based on these findings, as well as the study of the crystal structure of HN in complex with zanamivir\(^9\) and the reported study that led to the synthesis of the BCX compounds\(^10\), it was rationalised that Neu5Ac2en derivatives with bulkier substituents in C-4 and C-5 positions could be better-accommodated by HN and lock open the flexible loop to enhance inhibitor potency.

The study presented in Section 2.2 of this Chapter and published in Guillon et al.\(^11\), reports the structure-based design of potent inhibitors of HN based on derivatives of Neu5Ac2en that possess bulky phenyltriazole groups in C-4, and acetamido or butyramido moieties at C-5. The rational design of the inhibitors was assisted by extensive MD simulations, which led to the synthesis of a variety of compounds. They were discriminated by their ability to inhibit the HN neuraminidase activity, and were also evaluated for haemagglutination inhibition as well as inhibition of infection \textit{in vitro}. The binding epitope of the lead compound to HN, namely compound \(10\), was analysed by STD-NMR spectroscopy, using purified virus particles as well as insect cell-derived recombinant hPIV-3 HN expressed in a baculovirus system. From these experiments, the binding epitope of compound \(10\) to HN was established to facilitate the subsequent development of structurally related HN inhibitors.

Drug repositioning is an attractive approach in drug discovery, as it allows the selection of biologically-active compounds for a new purpose, that possess pre-established pharmacological properties\(^12\). Combined with target-based discovery, it makes a powerful tool in the identification of novel drug candidates whose mechanism of action is known, in a short time-frame.

The study reported in Section 2.3 of the present Chapter attempted to identify novel inhibitors of hPIV-3 HN, whose chemical structure are not necessarily related to sialic acid, by enzyme-based screening of an approved drug library on the sialidase function of HN. It was rationalised that such inhibitors, while unlikely to bind the active site of HN, would be inclined to act on the enzyme by allosteric modulation. They would therefore be likely to work in synergy with competitive inhibitors of the protein to block its function and infection \textit{in vitro}, thus providing a new direction for hPIV-3 combinatorial therapies.

The screening that was performed led to the selection of a single drug-hit, suramin,
that was also evaluated for its ability to inhibit the haemagglutination function of HN. It was tested in infection assays in vitro, and time-of-addition experiments were performed to understand the mode of action of the drug. Enzyme kinetics analysis, together with competition STD-NMR experiments in presence of the competitive inhibitor zanamivir, were used to determine the nature of the inhibition induced by the hit, namely to determine whether it was a competitive inhibitor of HN or not. Suramin was found to be a non-competitive inhibitor of the enzyme, and was further evaluated in in vitro infection assays in combination with zanamivir to assess if a synergistic effect could be observed between the drugs. These experiments were based on the method of Chou\textsuperscript{13,14}, which enables the evaluation of synergistic or antagonistic drug-induced effects by calculation of a combination index. As a proof of concept, the drug-hit was tested in combination with the novel low µM inhibitor of HN described in Section 2.2, to demonstrate its potential as a drug repurposable for combinatorial therapy applications. Finally, extensive in silico docking simulations were performed in an attempt to localise the binding site of the drug on HN.

Compound 10 represents a potent sialic acid-based inhibitor of the HN protein and serves as a lead compound for the design of more potent hPIV-3 drug candidates. Suramin, on the other hand, shows potential for combinatorial therapy against hPIV-3 infections and may set the basis for the development of a novel class of HN inhibitors.

References


2.2 Guillon et al., 2014

Statement of Contribution to Co-Authored Published Paper

This Section includes a co-authored paper. I am the second author on this paper as the first 4 listed authors are all joint first authors. The bibliographic details of the co-authored paper, including all authors, are:


My contribution to the paper involved:

1. The engineering and cloning of hPIV-3 HN, including the design and conception of experiments.
2. The acquisition and interpretation of data related to 1.
3. The review of the manuscript.

Permission to use the paper in this thesis has been obtained from Nature Publishing Group.

The Supplementary Material relevant to this paper can be found in Appendix 2, p. 193.

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<th>Student</th>
<th>Corresponding Author</th>
<th>Supervisor</th>
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<td>Benjamin Bailly</td>
<td>Prof. Mark von Itzstein</td>
<td>Prof. Mark von Itzstein</td>
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Human parainfluenza viruses (hPIVs) cause upper and lower respiratory tract disease in children that results in a significant number of hospitalizations and impacts health systems worldwide. To date, neither antiviral drugs nor vaccines are approved for clinical use against parainfluenza virus, which reinforces the urgent need for new therapeutic discovery strategies. Here we use a multidisciplinary approach to develop potent inhibitors that target a structural feature within the hPIV type 3 haemagglutinin-neuraminidase (hPIV-3 HN). These dual-acting designer inhibitors represent the most potent designer compounds and efficiently block both hPIV cell entry and virion progeny release. We also define the binding mode of these inhibitors in the presence of whole-inactivated hPIV and recombinantly expressed hPIV-3 HN by Saturation Transfer Difference NMR spectroscopy. Collectively, our study provides an antiviral preclinical candidate and a new direction towards the discovery of potential anti-parainfluenza drugs.
The Paramyxoviridae family contains viruses that are clinically significant in both medical and veterinary settings. Human parainfluenza viruses types 1 to 3 (hPIV-1, 2 and 3), members of this family, are the leading cause of upper and lower respiratory tract disease in infants and young children and also impact the elderly and immunocompromised. It is estimated that in the United States alone, up to five million lower respiratory tract infections occur each year in children who are below 5 years, and hPIV has been isolated in approximately one-third of these cases. There are currently neither vaccines nor specific antiviral therapy to prevent or treat hPIV infections, respectively. Some recent approaches have focused on an entry blockade and the triggering of premature virus fusion by a small molecule. A limited number of unsaturated neuraminic acid-based HN inhibitors (Fig. 1a) have been reported, although none of these inhibitors have advanced as clinical candidates presumably due to the lack of sufficient antiviral effect. One of the most potent and widely investigated hPIV type 3 haemagglutinin–neuraminidase (hPIV-3 HN) inhibitors, 6, has a neuraminidase IC₅₀ value of ~20 μM. This inhibitor incorporates an isobutyramido group at C5, demonstrating that hPIV-3 HN can accommodate larger acylamino groups in the C5-binding domain, and an azide functionality at C4 on the unsaturated neuraminic acid-based template.

We have recently described significant flexibility associated with the hPIV-3 HN 216 loop that borders the active site region. This flexible loop can establish a more open cavity, the 216 cavity, and provides new opportunities for inhibitor discovery. Here we successfully explore this exciting opportunity using a multidisciplinary approach that employs, structure-based computational chemistry, synthetic chemistry, NMR-based structural and biological techniques to discover potent inhibitors of hPIV-3 HN activity and cell infection.

**Results**

**Computational chemistry and inhibitor design.** The characterization of the hPIV-3 HN 216 loop flexibility motivated us to explore the potential of designing Neu5Ac2en derivatives with bulky C4 substituents that could be accommodated in and lock open the 216 cavity within the active site. Accordingly, molecular dynamics (MD) simulations have been employed to design and assess Neu5Ac2en derivatives that incorporate C4-functionalized triazoles. From our initial study of 216 loop flexibility and the resultant 216 cavity dimensions, it was clear that relatively bulky C4 substituents on the unsaturated neuraminic acid-based

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**Figure 1** | Structures of reference sialidase inhibitors, the novel hPIV inhibitors and the synthetic strategy. (a) Structures of N-acetylneuraminic acid (1), the naturally occurring sialidase inhibitor Neu5Ac2en (2), 4-azido-4-deoxy-Neu5Ac2en (3), the potent influenza virus sialidase inhibitor zanamivir (4), the CS isobutyramido analogue of Neu5Ac2en (5), the reference hPIV inhibitor BCX 2798 (6) and the novel inhibitors 7-10. (b) Synthesis of the novel hPIV inhibitors 7-10. (i) CuSO₄, sodium ascorbate, tert-butanol/H₂O (1:3), 45°C, 6 h; (ii) NaOH, MeOH/H₂O (1:3), RT, a/o (7, 85%; 8, 86%; 9, 92%; 10, 89%).
Compound 8 as an hPIV-3 HN inhibitor model. The simulation of the available hPIV-3 HN crystal structure (PDB accession code 1V3E) (ref. 25) in complex with 8, using a defined parameter set (Supplementary Table 1a–e), allowed an analysis of the dynamic behaviour of the protein relative to the zanamivir (4)-bound structure. Atom-positional root mean square deviations (r.m.s.d.) of the hPIV-3 HN backbone atoms (C, N, C) for the 216 loop from the simulations of the hPIV-3 HN–4 and –8 complexes are presented in Fig. 2a. From this study, it is apparent that the 216 loop undergoes more significant deviations from the crystal structure in the case of the hPIV-3 HN–8 complex (black curve). r.m.s.d. values greater than 0.5 nm are observed for the simulation of the hPIV-3 HN–8 complex, whereas the structure deviates less (0.4 nm) for the hPIV-3 HN–4 complex.

This notion is further supported by the root mean square fluctuations (r.m.s.f.) observed for the C8 atoms of the backbone for residues associated with the 216 loop (residues 205–225, Fig. 2b). Increased r.m.s.f. are observed for the residues of the second half of the 216 loop (215–220), where values of ~0.3 nm are reached, indicating a substantial conformational rearrangement within that domain compared with the starting hPIV-3 HN reference X-ray crystal structure (PDB accession code 1V3E) (ref. 25), Table 1 shows a selection of r.m.s.f. values of residues comprised in the 216 loop.

Our data suggest that loop flexibility, present under physiological simulation conditions, has been significantly underestimated in crystal structures and provides an exciting opportunity for anti-parainfluenza virus drug discovery. Comparison of the hPIV-3 HN–4 complex and the hPIV-3 HN–8 complex simulations demonstrates that the C8 substituent on 8 induces significant movement in the hPIV-3 HN 216 loop. The induced loop opening can be seen from the solvent-accessible surface plots of the final structures obtained from 10-ns simulations of hPIV-3 HN–4 complex (Fig. 2c, left panel) and hPIV-3 HN–8 complex (Fig. 2c, right panel).

One of the most populated conformational clusters from the MD simulations of hPIV-3 HN in complex with 4 and 8, are shown in Fig. 3a, while the superposition of the final conformations from the simulations of hPIV-3 HN in complex with 4 and 8 are shown in Fig. 3b. The difference in 216 loop conformation can clearly be seen (coloured loops, 1V3E–4 (magenta) and 1V3E–8 (green)). The 216 cavity adopts a more open conformation when in complex with the more sterically encumbered inhibitor 8. Generally, a wider cavity is observed for the simulation of hPIV-3 HN–8 complex. The most populated cavity from the simulation of the hPIV-3 HN–4 complex has a slightly smaller cavity volume (654 Å3) compared with the simulated hPIV-3 HN–8 complex (717 Å3).

To evaluate if a bulkier C8 acylamino moiety would be accommodated in the presence of the C4-functionalized triazole, we undertook an identical analysis of 10 in complex with hPIV-3 HN (Supplementary Movie 1). Our preliminary analysis led us to conclude that a C5 isobutyramido moiety is well accommodated within the C5-binding domain in the presence of the C4-functionalized triazole (Fig. 3c).
Inhibitor-relative interaction energies. To quantify the extent of inhibitor engagement with hPIV-3 HN, we have used our MD simulations approach to determine theoretical averaged interaction energies for the known inhibitor 2, based on a previous study\textsuperscript{24}, as well as the novel C5 acetamido and C5 isobutyramido inhibitors, 8 and 10, respectively. Average interaction energy (E\text{avI}) values of \(-609.38 \pm 10.92\), \(-733.96 \pm 15.49\) and \(-821.88 \pm 10.93\) kJ mol\textsuperscript{-1} for 2, 8 and 10, respectively, in complex with hPIV-3 HN (PDB accession code 1V3E) (ref. 25) were determined. Most importantly, these calculations support the notion that the replacement of the acetamido moiety in 8 with an isobutyramido moiety in 10 significantly improves the absolute E\text{avI} value of the inhibitor in complex with the protein. Consequently, 10 is predicted to be a more potent hPIV-3 HN inhibitor than 8. Further analysis of the MD simulation and extraction of the lowest (1,078.13 kJ mol\textsuperscript{-1}) interaction energy structure of 10 in complex with hPIV-3 HN (1V3E) revealed that 10 makes several key interactions, in this orientation, within the binding pocket (Fig. 3d). Noteworthy is the electrostatic interaction between the ligand’s carboxylate and the triarginyl cluster (Arg192, Arg424, Arg502) (Table 1).

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Figure 3 | The predicted structure of the HN active site in complex with 4 and 8. (a) Solvent-accessible surface plots of the most populated conformational clusters of hPIV-3 HN (1V3E) bound to 4 (left) and bound to 8 (right). The residues comprised in the 216 loop (205–225) are coloured in magenta (1V3E–4) and green (1V3E–8), respectively. Arginines associated with inhibitor binding are coloured in blue. (b) Superposition of snapshots of the final trajectories of hPIV-3 HN bound to 4 and 8 after 10 ns of MD simulation. The 216 loop is highlighted in colour (green: 1V3E–8, magenta: 1V3E–4). 4 (magenta) and 8 (atomic colours) are shown in stick representation. (c) Solvent-accessible surface plot of the final coordinates of hPIV-3 HN (1V3E) bound to 10. Both the C4-functionalized triazole and the C5 isobutyramido moiety are well accommodated in an open 216-loop structure of hPIV-3 HN. (d) Key interactions between 10 and hPIV-3 HN. The triarginyl cluster (Arg192, Arg424, Arg502) engages the inhibitor’s negatively charged carboxylate, the C7 hydroxyl group engages Glu276 in a hydrogen bond interaction as does the C5 isobutyramido NH with Tyr337 and Glu409. Hydrophobic interactions are observed for both the C4 aromatic and C5 isobutyl functionalities.
Arg502), hydrogen bond interactions between the C7 hydroxyl group and Glu276 and the C5 isobutyramido NH and Tyr337 and Glu409. Furthermore, additional hydrophobic interactions are observed for both the C4 aromatic and C5 isobutyl functionalities, particularly with the peptide backbone, within the C4- and C5-binding domains, respectively.

Synthesis of the target triazoles 7–10. Guided by our computational analysis, we synthesized the identified C4-functionalized triazole unsaturated N-acetylneuraminic acid derivatives 7–10 to further explore their capacity to inhibit hPIV-3 HN function by accessing an open 216 loop within the hPIV-3 HN-binding site. The synthesis of the triazoles 7–10 was achieved using the known26–29 4-azido-4-deoxy-Neu5Ac2en-based intermediates 11 and 12. Thus, each of the two intermediates was exposed to either methylpropanol ether or ethynylbenzene under typical click azide–alkyne coupling conditions37,38 (heating a mixture of the 4-azido-4-deoxy-Neu5Ac2en derivative, alkyn, CuSO4 and sodium ascorbate in a (1:1) mixture of water and tert-butanol for 6 h) to afford the triazole derivative (Fig. 1b).

Triazoles 13 and 14 (starting from 11) and the triazole derivatives 15 and 16 (starting from 12) were isolated in yields of 78, 82, 71 and 84%, respectively. The resulting per-O-acetylated triazole derivatives 13–16 were then deprotected by treatment with aqueous methanol (50%) adjusted to pH 13–14 at room temperature (RT) for 24 h to yield the final products 7–10 as sodium salts in 85, 96, 92 and 89% yields, respectively. The novel compounds were appropriately characterized (Supplementary Methods) and all compounds were determined, by 1H and 13C NMR spectroscopy, to be of high purity (Supplementary Fig. 3a–t).

Inhibitor screening with hPIV-3 HN. The reference inhibitors 2, 3, 5, 6 and 7, as well as the novel inhibitors 7–10, were evaluated for their capacity to inhibit both the neuraminidase and haemagglutinin functions of the hPIV-3 HN protein using neuraminidase inhibition (NI) and haemagglutination inhibition (HI) assays. Thus, NI was assessed by an end point fluorescence-based assay employing the substrate 2-((4-methylumbelliferyl) α-2-N-acetylneuraminic acid (MUN)39,40–42. The HI evaluation methodology43 utilized both guinea pig red blood cells (gp RBC) and human red blood cells (h RBC) to determine virus-mediated agglutination of erythrocytes.

A comparison of the potency of the synthesized C4-modified Neu5Ac2en derivatives against hPIV-3 HN was undertaken and, for convenience sake, the IC50 values were divided into two groups based on the acylamino group present at C5. Group 1 inhibitors have a C5 acetamido functionality and Group 2 inhibitors have a C5 isobutyramido functionality (Fig. 4, Supplementary Fig. 4 and Supplementary Table 2). The benchmark and well-characterized broad spectrum neuraminidase inhibitor Neu5Ac2en (2) showed the weakest inhibition with IC50 values of 1,565 μM and 1,438 μM for hPIV-3 HN NI and HI, respectively. The inhibition observed for 3, the C5 acetamido analogue of BCX 2798 (6), was improved when compared with 2, although it was still in the high micromolar range with IC50 values of 138 μM and 210 μM for hPIV-3 HN NI and HI, respectively. These IC50 values were similar to those observed for our novel inhibitor 7, a C4 methoxymethyl-functionalized triazole Neu5Ac2en derivative, with experimentally determined hPIV-3 HN NI and HI IC50 values of 154 and 313 μM, respectively. A significant improvement in potency was observed on replacement of the C4 triazole’s methoxymethyl moiety (7) with a bulkier phenyl group (8). IC50 values of 6.5 and 4.6 μM were determined for hPIV-3 HN NI and HI for 8, respectively.

Cell-based assays. Following initial enzymatic screening, the most potent inhibitor 10 and the reference hPIV inhibitor (BCX 2798, 6) were then evaluated in a growth inhibition assay to compare their capacity to inhibit hPIV-3 virus infection and propagation in LLC-MK2 cells (Supplementary Fig. 6). Compound 6 was chosen as a reference inhibitor as it is the most documented hPIV-3 Neu5Ac2en-based inhibitor to date and has reasonable
in vitro hPIV-3 antiviral potency\textsuperscript{18,21}. In an initial assay, at an inhibitor concentration of 2 μM, the virus was propagated for 48h in the presence of 6 or 10 and virus titres were determined. At this inhibitor concentration, a reduction of 14% and 94% in virus titre by 6 and 10, respectively, was calculated (Supplementary Fig. 6b). Virus growth inhibition IC\textsubscript{50} values were then determined for the two inhibitors in a well-established in situ enzyme-linked immunosorbent assay (ELISA) technique\textsuperscript{11} using three different cell lines. We chose LLC-MK2 (monkey kidney epithelial cells) cell line as it is extensively used in hPIV-3 cell-based infection studies\textsuperscript{13} as well as the hPIV-3 susceptible human respiratory cell lines A549 (lung adenocarcinoma epithelial cells)\textsuperscript{16} and normal human bronchial epithelial primary cells to investigate virus growth inhibition in natural tissue-related cells. The method itself has useful advantages over the virus titration method as it is a faster, one-step, non-subjective technique that correlates non-immobilized virus growth to HN expression levels of a low multiplicity of infection-infected cell monolayer. Interestingly, slightly lower virus growth inhibition IC\textsubscript{50} values were determined for 10 and 6 with the laboratory-established cell line LLC-MK2 in relation to the human cell lines. Overall, the same trend is observed for all the three cell lines in that a significantly stronger antiviral effect of inhibitor 10 (IC\textsubscript{50} = 2.1–13.9 μM) is determined compared with inhibitor 6 (IC\textsubscript{50} = 54.6–130.6 μM) (Fig. 5).

**Structural biology.** Saturation Transfer Difference (STD) NMR experiments of 8 in complex with recombinantly expressed hPIV-3 HN (Supplementary Fig. 7) and the most potent inhibitor 10 in complex with either recombinantly expressed hPIV-3 HN (Fig. 6) or intact hPIV-3 virus (Supplementary Fig. 8) were undertaken to further support our computational and biological studies that demonstrated specific binding and inhibition.

STD NMR signal intensities for all protons associated with 8 or 10 were clearly observed, to varying extents, when the inhibitor is in complex with either intact virus or recombinant hPIV-3 HN and clearly demonstrated that the ligand binds in both instances. The minor signals visible at 3.25, 3.5 and 4.0 p.p.m. in the \textsuperscript{1}H NMR spectrum of 10 acquired in the presence of intact virus particles were a consequence of impurities from the virus purification process and belong to neither the virus particles nor 10. As anticipated, none of these signals were observed in the STD NMR spectrum and clearly demonstrate that the impurities do not bind to the virus (Supplementary Fig. 8).

These experiments clearly demonstrate the specific binding of 10 to both intact hPIV-3 virus and hPIV-3 HN, further substantiating the inhibitor’s biological relevance and potential.

Importantly, an overlay of the aromatic phenyl protons signals observed at 7.1–7.6 p.p.m. in the STD NMR spectra for both the intact virus and recombinant HN protein also reveals that the binding epitope of inhibitor 10 is similar, if not identical, when bound either to intact hPIV-3 virus or to recombinant hPIV-3 HN protein (Supplementary Fig. 9).

**Epitope mapping of inhibitor 10.** A complete ligand-binding epitope was determined by the analysis of STD NMR spectra (Fig. 6) of hPIV-3 HN protein in complex with 10. All STD NMR signals of 10 were normalized to the strongest STD NMR signal observed, the inhibitor’s H4 proton at 7.18 p.p.m. Relative STD NMR effects for all protons of the inhibitor were then calculated (Supplementary Table 3). The extent of the STD NMR signal intensity strongly depends on the proton’s proximity to the protein surface and reveals how the designed inhibitor 10 engages the HN protein’s binding site.

Notably, very strong relative STD NMR effects were observed for the phenyl group protons H2, H3, H4, H5 and H6 between 7.1 and 7.6 p.p.m. revealing a close contact in that region of the molecule to the protein surface. Moreover, a significant STD NMR effect was likewise detected for the CH of the triazole moiety. In contrast, the C5 isobutyramido moiety’s protons of the inhibitor showed less effect (relative STD NMR signal intensities in the range of 42–54%).

Interestingly, the protons associated with the NeuAc2en core structure of 10 displayed variable relative STD NMR effects. A significant H3-relative STD NMR signal intensity (80%) suggests a strong interaction of this part of the molecule with hPIV-3 HN. Furthermore, relative STD NMR signal intensities for H4, H5 and H6 of 59, 50 and 49%, respectively, demonstrate that the ring protons of the NeuAc2en core structure are also involved in inhibitor engagement to the protein.

Finally, weaker relative STD NMR effects of 36, 35, 24 and 21%, were observed for the glycerol side chain protons H7, H8, H9 and H9’, respectively, and suggest that the glycerol side chain makes less of a contribution to the inhibitor-binding event compared with the C4 triazole functionality and the inhibitor’s core ring structure (Fig. 6).

The inhibitor 8 epitope map (Supplementary Fig. 7) was for all intents and purposes identical to that of inhibitor 10, with the C4 triazole moiety clearly in close contact to the protein surface.

**Discussion.** We have utilized MD simulations to verify the possibility, and to design and study the effect, of accommodating a NeuAc2en-based inhibitor with a bulky C4 substituent in the binding pocket of hPIV-3 HN. Previous MD studies\textsuperscript{22} on this protein have demonstrated the existence of a flexible loop, the 216 loop, which can adopt an open conformation creating a larger cavity in the region of the NeuAc2en C4 hydroxyl group. Our detailed MD simulations in the current study clearly demonstrate that substantially larger substituents at the C4 position of NeuAc2en...
in the presence of 20 (calculated and confirms the positive influence on neuraminidase inhibitor 2
such C4-functionalized inhibitors is highly efficient and should functionality occupying the 216 cavity and locking open the 216 accommodation of bulkier C4 substituents is achieved by the C4 (Figure 6 |1H and STD NMR spectra and epitope map of 10 in complex with hPIV-3 HN. (a) 1H NMR spectrum of 10 in the presence of 20 μM hPIV-3 HN at a protein:ligand ratio of 1:100 (2 mM of 10). (c) 1H NMR spectrum of the H7, H8, H9 and H9′ regions. (d) STD NMR spectrum of the H7, H8, H9 and H9′ regions. (e) Proposed binding epitope map of inhibitor 10.

(2) may be accommodated in the hPIV-3 HN’s active site. The accommodation of bulkier C4 substituents is achieved by the C4 functionality occupying the 216 cavity and locking open the 216 loop. Furthermore, our simulations predict that the binding of such C4-functionalized inhibitors is highly efficient and should result in potent inhibition of hPIV-3 HN. For example, a ΔE216 of –212.50 kJ mol⁻¹ between 10 and the extensively studied neuraminidase inhibitor 2 in complex with hPIV-3 HN was calculated and confirms the positive influence on E216 for the combined C4/C5 modification on the Neu5Ac2en template.

To experimentally validate our theoretical predictions, we successfully synthesized, in less than 10 steps, the designed combined C4/C5 modification on the Neu5Ac2en template. The biological evaluation of the reference and novel designer C4- and C5-functionalized Neu5Ac2en inhibitors using protein-commercially available starting materials in very good overall yield. Our synthetic strategy towards these novel inhibitors provides great versatility for additional functionalization and enables further exploration of the Neu5Ac2en template in inhibitor optimization studies.

The biological evaluation of the reference and novel designer C4- and C5-functionalized Neu5Ac2en inhibitors using protein-based inhibition assays (NI and HI) led to a number of conclusions concerning the influence of C4 and C5 substituents on inhibitor potency. Within each of the two screened groups, that is Group 1 (C5 acetamido) and Group 2 (C5 isobutyramido), the order of potency based on the substituent at C4 was found to be as follows: hydroxyl< azido≤ 4-methoxymethyltriazole< 4-phenyltriazole. The weakest inhibition in both groups was observed for the 4-hydroxy derivatives 2 and 5. This outcome supports the notion that the C4-binding domain, which accommodates the C4 hydroxyl group on Neu5Ac2en (2), has significant hydrophobic character and consequently does not favour the interaction with a polar, hydrophilic group including a hydroxyl group. The hydrophobic nature of the pocket, combined with the large 216 cavity size created by the opening of the 216 loop, does favour inhibitors, including inhibitors 8 and 10, which have the C4 hydroxyl group replaced with bulky hydrophobic substituents.

Comparison of both group’s IC50 values (Fig. 4) revealed that replacement of the C5 acetamido group with an isobutyramido group in all of the prepared inhibitors led to overall enhanced potency. Typically, close to an order of magnitude improvement was observed, except for the most potent inhibitor 10. Furthermore, analysis of the IC50 values (Fig. 4) supports the notion that the potency enhancement in the best inhibitors, 8 and 10, results predominantly from the introduction of the C4 substituent, with the C5 substituent contributing to a much lesser extent. This notion is also substantiated by STD NMR data analysis (Fig. 6) that led to an epitope map of inhibitor 10 in which the protons of the 4-phenyltriazole moiety showed the strongest contribution to the binding event of 10 in complex with hPIV-3 HN, while the relative interactions observed for the isobutyramido group were significantly less (~50%).

Further comparison of the various groups of inhibitors provided relative IC50 improvement factors for both NI and HI as detailed (Supplementary Fig. 4). Interestingly, a comparison of the parent templates 2 and 5, which contain a C4 hydroxyl group, with their corresponding C4-functionalized derivatives 8 and 10, respectively, shows dramatic HI improvement on C4 functionalization. The trend in the increase in NI is similar to that observed for HI; for example, inhibitors 5, 6 and 9 had a 5–20-fold enhancement in potency by increasing the size of the C5 substituent from an acetamido to an isobutyramido functionality. However, a comparison of the potential NI and HI improvement factors also suggests that when a bulky C4 substituent is present, as in 8 and 10, the introduction of the bulkier C5 isobutyramido moiety (10) improves potency to a lesser extent, by only a factor of two to three compared with the acetamido moiety (8). The consequence of locking the 216 cavity open by the bulky...
C4-functionalized derivative is positional changes of amino acid side chains associated with the active site domains that recognize the C4 and C5 functionalities on N-acetylneuraminic acid (1) and its various unsaturated derivatives. These changes appear to equally accommodate the smaller C5 acetamido substituent and the bulkier C5 isobutyramido functionality.

The potent inhibition of both HN functions (NI and HI) by inhibitor 10 demonstrates that the compound exerts its antiviral effect against hPIV-3 by action on the virus’ key HN protein. These findings are further supported by STD NMR experiments of 10 in complex with either the intact virus or the recombinant HN protein, which clearly show identical STD NMR signal intensities for the inhibitor’s C4 triazole aromatic moiety (Supplementary Figs 8 and 9). Moreover, the calculated binding epitope for 10 in complex with hPIV-3 HN (Supplementary Table 3) is in excellent agreement with our MD simulations that clearly predict the close contact of the Neu5Ac2en derivative’s H3 and the C4 triazole moiety’s phenyl protons to the protein surface (Fig. 3). These results (Fig. 5) were in good agreement with the NI and HI assay data. Interestingly, the LLC-MK2 cell-based assays demonstrated that 10 is even more potent at the cellular level compared with NI and HI protein-based assays. In this cell-based assay, 10 was found to be ~26 times more potent than 6, whereas protein inhibition assays showed only ~8 and 11-fold improvement in NI and HI assays, respectively (Supplementary Fig. 4). This strongly suggests that 10 is a potent dual-acting inhibitor that derives efficient synergism from the inhibition of both the protein’s neuraminidase and haemagglutinin activities. This is in contrast to the previously reported inhibitor 6, which derives less synergistic effect as a result of its significantly poorer inhibition of the haemagglutinin activity. Finally, the extent of virus growth inhibition in both human cell lines for inhibitor 10 compared with 6 clearly demonstrates the superiority of the designer ligand 10 (Fig. 5).

In conclusion, we have taken advantage of the hPIV-3 HN 216 loop flexibility14. On the basis of the notion that the 216 cavity, created by loop movement, is of sufficient size to accommodate larger functionalities at C4 on the Neu5Ac2en-based template, we designed and synthesized inhibitors 7–10 that incorporate a bulky substituted triazolo substituent at C4. Our predicted efficient virion progeny release and provide new direction towards anti-HN dual-acting inhibitor development. Finally, we have found that these inhibitors efficiently block both virus cell entry and virion progeny release and provide new direction towards anti-parainfluenza drug development.

**Methods**

**MD simulations.** MD simulations were performed with GROMOS software27–29 using the force-field parameter set 54A4 (ref. 39). Initial coordinates were taken from the X-ray structure (PDB accession code 1V3E) (ref. 25) of hPIV-3 HN in complex with a 100% homology model of the 216 loop (Supplementary Table 1a–e). The number of atoms in the final composite system for 1V3E–C0 was 11,77,454, corresponding to 45 atoms from the crystal structure. Parameters for 8 were generated in an analogous manner38 to existing parameters in the GROMOS force field (Supplementary Table 1a–e). The number of atoms in the final composite system for 1V3E–4 and 1V3E–8 was 78,253 and 78,084, respectively. Ionization states of amino acid residues were assigned at pH 7.0. The histidine side chains were protonated at the Nε atom, except for His30 and His32. With the X-ray structure as a template, water molecules were added around the protein within a truncated octahedron with a minimum distance of 1.4 nm between the protein atoms and the square walls of the periodic box. All bonds were constrained with a geometric tolerance of 10−4 using the SHAKE algorithm40.

A steepest descent energy minimization of the systems was performed to relax the solute–solvent contacts while positionally restraining the solute atoms using a harmonic interaction with a force constant of 104 kJ mol−1 nm−2. Next, steepest descent energy minimization of the system without any restraints was performed to eliminate any residual strain. The energy minimizations were terminated when the energy change per step by one simulation step was ~0.1 kJ mol−1 nm−2. For non-bonded interactions, a triple-range method with cutoff radii of 0.8/1.4 nm was used. Short-range van der Waals and electrostatic interactions were evaluated at each time step based on a charge group pair list. Medium-range van der Waals and electrostatic interactions, between (charge group) pairs at a distance longer than 0.8 nm and shorter than 1.4 nm, were evaluated every fifth time step, at which point the pair list was updated. Outside the longer cutoff radius, a reaction-field approximation35 was used with a relative dielectric permittivity of 78.5. The centre of mass motion of the whole system was removed every 3,000 time steps. Solvent and solute were independently weakly coupled to a temperature bath of 295 K with a relaxation time of 0.1 ps (ref. 44).

The systems were also weakly coupled to a pressure bath of 1 atm with a relaxation time of 0.5 ps and an isothermal compressibility of 0.7513 × 10−5 (kJ mol−1 nm−2)−1. MD simulations of 20 ps periods with harmonic position restraining of atoms and force constants of 5 × 104, 2.5 × 104, 2.5 × 104 and 2.5 × 104 kJ mol−1 nm−2 were performed to further equilibrate the systems at 50, 120, 180 and 300 K, respectively. The simulations were each carried out for 30 ns. The trajectory coordinates and energies were saved every 0.5 ps for analysis.

Simulation trajectories for hPIV-3 HN in complex with 8 were produced in an analogous manner to that described above41 and were used for analysis and comparison of results obtained for hPIV-3 HN in complex with 8.

Analyses were done with the analysis software GROMOS ii (ref. 45). Atom-positional r.m.s.d.s between structures were calculated for the residues comprising the 216 loop (residues 210–221) by performing a rotational and translational atomic-positional least-squares fit of one structure on the second (reference) structure using a given set of atoms (N, Cα, C).

Atom-positional r.m.s.f.s were calculated as an average from a 30–ns period of simulation by performing a rotational and translational atomic-positional least-squares fit of the Cα atoms of the trajectory structures on the reference. r.m.s.f.s were calculated for all residues including the 216 loop (residues 210–221) with a reduced, representative structural ensembles for the simulations, r.m.s.d.-based conformational clustering was performed26,48. Structures extracted every 10 ps from simulations were superimposed on backbone-Cα atoms to remove overall rotation and translation. Clustering of all atoms of residues that line the binding site (residues 190–198, 210–221, 251–259, 274–280, 310–316, 334–339, 369–407, 413, 471–478, 529–537) was performed to compare relative structural populations of hPIV-3 HN protein from the different simulation trajectories. The similarity criterion applied was the r.m.s.d. of all of these residues with a cutoff of 0.13 nm. Final structures resulting from the 30 ns of MD simulations were extracted. Interaction energies between hPIV-3 HN and inhibitors 8 and 10 were calculated using GROMOS generated energies, free-energy i derivatives and block averages as separate trajectory files, referred to as the energy trajectory49. The programme enc_and45 was used to extract individual interaction energy values and the contributions, that is, van der Waals and Coulomb interactions from these files. Thus, these contributions between the ligand and the protein atom pairs corresponding to the energy trajectory resulting from the simulation and interaction energies calculated. The error estimate was calculated from block averages of growing sizes extrapolating to infinite block size. Hydrophobic interactions were analysed and a map of interactions between inhibitor 8 and hPIV-3 HN was created using LIGPLOT47. To measure the extent of cavity opening for selected structures, the pocket volume was analysed using POVM48. Importantly, extended simulation times, up to 80 ns provided outcomes entirely consistent with the data presented.

**Chemistry.** Synthesis of intermediates. The synthesis of intermediates (ref. 28), (ref. 29) and (ref. 28) and reference inhibitors (ref. 29, 49, 8, ref. 50, 2, (ref. 29) and 6) was achieved by the relevant literature procedure. General methods are described in the Supplementary Methods.

**General procedure for the synthesis of 18 and 19.** A mixture of 17 or 11 (0.42 mmol), p-BOC (275 mg, 1.27 mmol) and 3-Dimethylaminopropionitrile (50 mg, 0.42 mmol) in anhydrous tetrhydrofuran (5 mL) was stirred under argon atmosphere at 60°C (Supplementary Fig. 1). After cooling to RT, the solvent was evaporated under vacuum, and the residue was taken up in dichloromethane (DCM) for chromatographic separation on a silica gel column. The acetate hexahydrofuran (1,2,3) as solvent to yield pure 18 (170 mg, 71%; 19 (225 mg, 96%).

**General procedure for the synthesis of 20 and 21.** To a methanolic solution of NaOMe, freshly prepared by dissolving sodium (0.39 mmol, 9.8 g, 0.75 M) and anhydrous MeOH (5 mL), was added compound 18 or 19 (0.26 mmol) (Supplementary Fig. 1). The mixture was stirred at RT for 1 h and then quenched with Amberlite IR-120 (H+) resin (to pH=5). The resin was filtered off, washed

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with MeOH (5 ml x 3) and the combined filtrate and washes were evaporated under vacuum. The residue was redissolved in pyridine (2 ml), and acetic anhydride (5 ml) added. The mixture was then stirred at RT under argon atmosphere o/n and the solvent and excess Ac₂O were then removed under vacuum. Finally, the residue was taken up in DCM for chromatographic separation on a silica gel column using ethyl acetate/benzene (1:1) as solvent to yield pure 24 (112 mg, 81%) or 25 (84 mg, 63%).

General procedure for the synthesis of 22 and 23. To a solution of 20 or 21 (0.15 mmol) in anhydrous DCM (2 ml) was added TFA (1.20 ml, 15 mmol) and the mixture was stirred at RT under argon o/n and the solvent and excess Ac₂O were then removed under anhydride (0.5 ml) added. The reaction mixture was stirred at RT under argon o/n (Supplementary Fig. 1). The reaction was diluted with DCM (20 ml) and quenched with saturated aqueous Na₂CO₃ solution (20 ml). The DCM layer was washed with water and brine and then dried over anhydrous Na₂SO₄. The dried organic solvent was concentrated under vacuum.

General procedure for the synthesis of 5 and 6. To a suspension of compound 24 or 25 (0.88 mmol) in a 1:1 mixture of MeOH/H₂O (2 ml) at 0 °C was added dropwise a NaOH solution (1.0 M) until pH ~ 14 (Supplementary Fig. 1). The temperature was raised gradually to RT and the mixture was stirred at RT overnight. The solution was then acidified with Amberlite IR-120 (H⁺) resin (to pH=3), filtered and washed with MeOH (10 ml) and H₂O (10 ml). The combined filtrate and washes were then concentrated under vacuum and the residue was diluted with distilled water (5 ml) and adjusted to pH=8.0 using 0.05 M NaOH to convert the compound to its sodium salt. The compounds were then purified on a C18-Grace cartridge using 2% acetonitrile/water as solvent to yield 5 (26 mg, 94%) or 6 (24 mg, 82%) as white fluffy powders.

General procedure for the synthesis of 3-10. The 4-azido-4-deoxy-NeuAcα2-2-sialyllactose (0.010 mmol) and the corresponding sialyl ceramide derivative (0.033 mmol) were dissolved in a 1:1 mixture of tert-butanol/H₂O (4 ml). Compound 21 and 22 were added (0.1 M) and the mixture was stirred at 45 °C for 15 min. Inocula were removed and replaced with 500 µl of each well of each respective 2 µM compound dilution (in EMEEM). A positive control for infection was included using the same conditions minus the compound. Virus proliferation on infected cell monolayers was measured for 48 h at 37 °C.

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Clariﬁed hPIV-3 supernatant was mixed with PEG6000 (8% ﬁnal concentration) and NaCl (0.4 M ﬁnal concentration) and then incubated overnight at 4 °C under gentle agitation. PEG6000/hPIV-3 complex was pelleted by centrifugation at 3,000 x g for 30 min at 4 °C. The supernatant was discarded and a volume of GNTE buffer (glycine, 200 mM, NaCl, 200 mM, Tris-HCl, 20 mM, EDTA, 2 mM, pH 7.4) corresponding to at least 1/4 of the initial virus suspension volume was used to resuspend the pelleted virus overnight at 4 °C. The virus suspension was homogenized by up and down pipetting followed by a mechanical disruption of the remaining virus aggregates using a dounce with ‘tight’ pestle. The hPIV-3 homogenate was loaded on top of a 30–60% non-linear sucrose gradient prepared in GNTE buffer and centrifuged at 100,000 x g for 2 h 30 min at 4 °C without brake for deceleration. The virus band was then harvested and collected and stored at ~ 80 °C for NI assays or at 4 °C for STD NMR experiments.
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anti-hPIV-3 HN (Fitzgerald, clone# M02122321, 2.0 mg ml  1) at 1 mg ml  1 in 5%
milk/PBS for 1 h at 37 °C. The wells were washed three times for 5 min with 0.02%
Tween20/PBS. Goat anti-Mouse-IgG(H+L)-HRP conjugate (BioRad, ref# 170–
6516), diluted at 1:2,000 in 5% milk/PBS, was added to each well and incubated for
1 h at 37 °C. Cell monolayers were washed with 0.02% Tween20/PBS and then
rinsed twice with PBS. BD OptEIA TMB substrate (BD Biosciences, San Jose, CA,
100 ml) was added to each well and the plate was then incubated at 37 °C. The
enzymatic reaction was stopped after 3–5 min by the addition of 50 ml of 0.6 M of
H2SO4 per well. Raw data were obtained by reading the absorbance (OD) of each
well at 450 nm for 0.1 s with a Victor 3 multilabel reader (PerkinElmer, Waltham,
MA). Final ODs were obtained by subtraction of the negative control (non-infected
cells) OD from the initial OD reading and the data analysed with GraphPadPrism4
(GraphPad Software, Inc., La Jolla, CA) to calculate IC50 values (non-linear
regression (curve ﬁt), Dose–response inhibition, four-parameter logistic). The IC50
value was considered as the concentration of inhibitor that reduced the absorbance
at 450 nm by 50%, compared with a non-treated infected cell monolayer.
Standard deviation calculations. Standard deviations were calculated with
GraphPadPrism4 (GraphPad Software, Inc., La Jolla, CA).

Structural biology. Sample preparation and 1H NMR experiments. All NMR
experiments were performed on a 600 MHz NMR spectrometer (Bruker) equipped
with a 5-mm TXI probe with triple axis gradients. Intact virus suspension or
recombinant hPIV-3 HN were buffer exchanged against 50 mM deuterated sodium
acetate, 5 mM CaCl2 in D2O at pD 4.6 by ultraﬁltration using an Amicon Filter
Unit (Millipore) with a cutoff value of 30 or 10 kDa, respectively. For each
experiment, 20 mM hPIV-3 HN protein and a protein:ligand molar ratio of 1:100 in
a ﬁnal volume of 200 ml was used.
1H NMR spectra were acquired with 32 scans at 283 K, a 2-s relaxation delay
over a spectral width of 6,000 Hz. An initial STD NMR experiment was performed
on a complex between compound 10 and intact hPIV-3 virus and all subsequent
STD NMR experiments were performed on compounds in complex with
recombinant hPIV-3 HN protein.
STD NMR experiments. The protein was saturated on-resonance at  1.0 p.p.m.
and off-resonance at 300 p.p.m. with a cascade of 60 selective Gaussian-shaped
pulses of 50 ms duration, resulting in a total saturation time of 3 s and the
relaxation delay was set to 4 s. Each STD NMR experiment was acquired either
with a total of 1,056 scans (recombinant hPIV-3 HN) or 1,512 scans (intact virus)
and a WATERGATE sequence was used to suppress the residual HDO signal. A
Spin-lock ﬁlter with 5 kHz strength and duration of 10 ms was applied to suppress
protein background. Control STD NMR experiments were performed with an
identical experimental setup and the same ligand concentration but in the absence
of protein. On- and off-resonance spectra were stored and processed separately,
and the ﬁnal STD NMR spectra were obtained by subtracting the on- from the
off-resonance spectra. All STD effects were quantiﬁed using the equation
ASTD=(I0  Isat)/I0=ISTD/I0. Therefore, signal intensities of the STD NMR spectrum
(ISTD) were compared with the corresponding signal intensities of a reference
spectrum (I0). The strongest STD signal in the spectrum was assigned to a value of
100% and used as a reference to calculate relative STD effects accordingly56–58.

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Chapter 2. Inhibitors of hPIV-3 Infection


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Author contributions

M.v.I. conceived and oversaw the project. I.M.E.-D. performed all of the described chemistry. M.W. performed all of the molecular dynamics simulations studies. P.G., L.D. and B.B. performed the biological experiments. J.C.D. contributed to the molecular modelling studies. P.G. and L.D. performed the STD NMR studies and T.H. provided advice and assisted in the analysis of these studies. All of the authors contributed to the design, analysis and discussion of the research and writing of the manuscript.

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Statement of Contribution to Co-Authored Published Paper

This Section includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

**Bailly, B., Dirr, L., El Deeb, I., Altmeyer, R., Guillon, P., von Itzstein, M.** Suramin is a non-competitive inhibitor of hPIV-3 hemagglutinin-neuraminidase that acts in synergy with zanamivir to block hPIV-3 infection *in vitro*. Manuscript under internal review, intended for submission to PLoS Pathogens.

My contribution to the paper involved:

1. The conception and design of the study.
2. The acquisition and interpretation of most data.
   - Haemagglutination inhibition experiments were performed by Dirr, L.
   - The production of purified hPIV-3 was performed by Guillon, P.
   - The synthesis of Compound 10 and Zanamivir was performed by El Deeb, I.
3. The preparation of all figures.
4. The preparation of the draft manuscript.

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<th>Student</th>
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<td>Prof. Mark von Itzstein</td>
<td>Prof. Mark von Itzstein</td>
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Suramin is a non-competitive inhibitor of hPIV-3 haemagglutinin-neuraminidase that acts in synergy with zanamivir to block hPIV-3 infection in vitro.

Short title: Suramin inhibits HN and prevents hPIV-3 infection

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Abstract

Human parainfluenza type-3 virus (hPIV-3) is one of the principal aetiological agents of acute respiratory illness in infants worldwide. Although the virus also shows high severity towards the elderly and immuno-compromised, neither therapies nor vaccines are available to treat or prevent infection. In this study, we report the discovery of the approved drug suramin as an inhibitor of the hPIV-3 haemagglutinin-neuraminidase (HN), which displays strong synergism with zanamivir to inhibit infection in vitro. We identified suramin by enzyme-based, semi-high throughput screening of an approved drug library on the neuraminidase function of hPIV-3 HN. The drug was found to efficiently inhibit the neuraminidase with a 50\% inhibitory concentration (IC\textsubscript{50}) of 13 \(\mu\)M. In addition, the drug inhibited viral infection of mammalian epithelial cells in vitro with an IC\textsubscript{50} of 30 \(\mu\)M. Kinetic analysis of the neuraminidase with the substrate MUN in presence of drug suggested that suramin inhibits the enzyme via a non-competitive mechanism with a K\textsubscript{i} value of 4.9 \(\mu\)M; this finding was confirmed using purified hPIV-3 particles in competition STD-NMR experiments with suramin and the sialic acid-based inhibitor zanamivir. Most importantly, suramin in
Combination with zanamivir was found to be synergistic in cell-based drug-combination studies, suggesting that lower concentrations of both drugs can be used to yield high levels of inhibition. *In silico* docking simulations led us to the hypothesis that suramin inhibits the hPIV-3 HN by binding in the vicinity of the protein primary binding site, resulting in an enhancement of the inhibitory potential of sialic acid-based inhibitors.
Introduction

Human parainfluenza viruses (hPIV) are currently the second most prevalent cause of acute respiratory tract infection in infants after human respiratory syncytial virus (hRSV). Among parainfluenza viruses, hPIV-3 is the principal causative agent of disease in infants [1–3]. Apart from being mostly infectious towards children under 2 years of age, they also cause severe respiratory symptoms to the elderly, the immuno-compromised and transplant patients [4–6]. To date, neither drugs nor vaccines are available to either treat or prevent hPIV-3 infection.

hPIV-3 has two surface envelope glycoproteins, the haemagglutinin-neuraminidase (HN) and the fusion protein (F). While F is responsible for fusion of the virus envelope with the host cell membrane, HN is responsible for attachment of the virus to sialylglycoconjugates on host cell receptors containing an α2-3 or α2-6-linked terminal sialic acid (N-acetylneuraminic acid) [7,8]. It is also responsible for cleavage of sialic acid entities to promote the release of virus progeny from the cell [1,9]. Additionally, it has a third role in the stabilisation of F and initiation of fusion [10–15]. HN has a key position in the replication cycle of the virus, and is considered as a valid target for antiviral drug discovery [16–19].

HN possesses a primary binding site, responsible for both receptor recognition and cleavage. A potential secondary binding site has besides been identified at the dimer interface, and has been shown to be involved with fusion [10,20]. As it is the case for hPIV-1 [21], hPIV-3 HN could also possess another secondary binding site, masked by an N-glycosylation at residue 523 [22,23].

Although HN primarily recognises and cleaves terminal sialic acids from glycoconjugates on host cell receptors, it can recognise larger sialic acid-containing glycan structures such as...
sialyl-Lewis^\text{x} [24] and sialyl-blood group I-type antigens [7]. Moreover, a report from Winger and von Itzstein [25] demonstrated, using a molecular dynamic simulations study, that the protein possesses a flexible loop around aspartic acid 216, in the vicinity of the active site. This loop is thought to be open in the apo-form of HN for the active site to accommodate substrates larger than the terminal sialic acid moiety of a sialoglycoconjugate alone, and to close upon receptor binding, confirming what has been previously shown for hPIV-3 HN [24].

Most of the compounds that are investigated as anti-hPIV-3 molecules are competitive inhibitors of HN. They are primarily sialylmimetics based on Neu5Ac2en (DANA, N-acetyl-2,3-didehydro-2-deoxy-neuraminic acid), one of the first inhibitors of a broad range of neuraminidases (also denominated sialidases) [26]. As such, they are derivatives of the influenza virus neuraminidase inhibitor zanamivir [27,28], or the hPIV inhibitor BCX 2798. The latter shows some prophylactic efficacy in a mouse model of hPIV-3 infection [17,29,30] (1-4, Figure 1). Literature suggests that compounds larger than sialic acid could bind to HN [25]; this particular feature of the protein was confirmed by the recent rational design and synthesis of a potent, bulky C4-substituted phenyltriazole derivative of Neu5Ac2en, named compound 10 (5, Figure 1) which was shown to be accommodated nicely into the active site and to lock the 216-loop open upon binding [19].

In the present study, we have investigated whether approved drugs, not related to sialic acid, could block the HN hemagglutination and neuraminidase functions. Such drugs would contribute to the discovery of novel ways to block HN, and would have the advantage to be potentially repositionable. Through this method, we have successfully identified suramin, a trypanocidal drug commonly prescribed for the treatment of sleeping sickness in Africa [31–33], as a non-competitive inhibitor of the receptor binding and neuraminidase functions of HN. Moreover, we have demonstrated that the drug has \textit{in vitro} antiviral potency and shows...
synergism when combined with competitive inhibitors of HN. Our study shows that compounds other than sialylmimetics can indeed inhibit neuraminidases by binding, in the case of the hPIV-3 HN, in a region of importance in close proximity to its active site.

**Figure 1.** Chemical structures of N-acetylneuraminic acid (1), Neu5Ac2en (2), zanamivir (3), BCX-2798 (4) and compound 10 (5) The positions of carbon atoms, conserved in 1-5, are numbered in blue on structure 1. Ph = phenyl, Ac = acetyl.
Materials and Methods

Cells and viruses. LLC-MK2 cells were provided by Institut Pasteur Shanghai’s Pathogen Diagnosis Centre and were maintained at 37 °C under a humid atmosphere of 5% CO₂ in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. The human parainfluenza type-3 virus (strain C-243) was obtained from the American Type Culture Collection (Manassas, VA) and propagated in LLC-MK2 cells in un-supplemented DMEM. Virus stocks were prepared by infecting cells at a low multiplicity of infection (MOI) of 0.1. After 3 days of incubation, the virus-containing culture supernatant was clarified by centrifugation at 2000 × g for 15 min at 4 °C, aliquoted and stored at -80 °C for subsequent titration by focus forming assay.

Compounds. Compound 10 and zanamivir were synthesised at the Institute for Glycomics, as previously described [19,34]. Neu5Ac2en (DANA, N-acetyl-2,3-didehydro-2-deoxyneuraminic acid) was purchased from J & K Chemical (Shanghai, China), and the neuraminidase substrate MUN (2-(4-methylumbelliferyl)α-D-N-acetylmuramid acid) was purchased from Sigma Aldrich (St. Louis, MO).

Enzymatic screening of hPIV-3 neuraminidase inhibitors. The USA-approved and International-approved drug libraries (1280 compounds) were purchased from MicroSource Discovery System, CT. The drugs were diluted in reaction buffer prior to testing in NI assay in duplicates, at a single concentration of 100 µM. The compound Neu5Ac2en (DANA, N-acetyl-2,3-didehydro-2-deoxyneuraminic acid), was used as a positive control of inhibition at a final concentration of 10 mM (2 mM IC₅₀ for hPIV-3’s HN [16]). A threshold of 80% of neuraminidase inhibition at 100 µM was chosen to discriminate hit compounds. The potency (IC₅₀) of the hits was then determined by dose-response experiments.
Neuraminidase inhibition assay. The anti-neuraminidase activity of compounds was evaluated by NI assay. To determine the optimal concentration of PEG-precipitated virus required for the assay, the neuraminidase activity of HN without inhibitor was tested beforehand, at fixed substrate concentration and varying enzyme concentrations. An enzyme dilution within the linear range of the dose-response curve was targeted, yielding at least 5 times the signal of background. In addition, the highest acceptable concentration of DMSO in the reaction was determined by testing different concentrations of the organic diluent, at fixed enzyme and substrate concentrations, in order to accurately evaluate hydrophobic compounds.

The assays were performed as described previously [19], using a modified protocol from Potier et al. [35], Suzuki et al. [36] and Holzer et al. [37]. PEG-precipitated virus and compounds were diluted respectively in GNTE buffer and reaction buffer (NaOAC 50 mM, CaCl₂ 5 mM, pH 4.6. Into the wells of black 96-well plates were combined 7 µL of reaction buffer, 1 µL of compound, and 1 µL of virus. The plates were pre-incubated for 30 min at 37 °C before 1 µL of the fluorogenic neuraminidase substrate MUN (2-(4-methylumbelliferyl)α-D-N-acetyleneuraminic acid) was added to the mixture at a final concentration of 2 mM. The plates were centrifuged briefly in a swinging-bucket rotor, and incubated at 37 °C for 30 min at 1000 rpm. To stop the reaction, 150 µL of carbonated buffer pH 10.8 was added in each well. The plates were read at an excitation wavelength of 335 nm and an emission wavelength of 460 nm in a Varioskan Flash Multimode Reader (Thermo Scientific). The Relative Fluorescence Units (RFU) values for each measurement were normalised to the blank (reaction stopped immediately after addition of substrate) and expressed as percentage of inhibition of hPIV-3 neuraminidase. The IC₅₀ for NI assay has been defined as the average inhibitory concentration of a compound leading to a 50% reduction of hPIV-3 HN neuraminidase activity.
Kinetic analysis. The inhibition mechanism of hit compounds was determined by enzyme kinetics experiments on the hPIV-3 HN protein using a modified protocol of Ryu et al. [38], Nguyen et al. [39] and Mishin et al. [23]. The neuraminidase activity of HN was measured every 2 min over a period of 20 min, at 5 concentrations of MUN [S]: 2, 4, 8, 10 and 20 mM, and 4 concentrations of inhibitor [I]: 0, 6, 10 and 16 µM. The initial velocity $v_i$ of the enzyme for each [S]—[I] combination was determined automatically by the software provided with the Varioskan Flash Multimode Reader. A Lineweaver-Burke plot was represented for all [I], by plotting $\frac{1}{v_i} = f(\frac{1}{[S]})$ and applying a linear regression using the software GraphPad Prism version 5 (GraphPad Software, La Jolla California USA). The point of convergence of the 4 linear regressions helped determine the nature of the compound inhibition (competitive, non-competitive, un-competitive, or mixed-inhibition), and allowed the calculation of the Michaelis-Menten constant $K_M$ of the enzyme for the substrate, as the intercept when $Y=0$ equals $-\frac{1}{K_M}$. The maximum velocity $V_{max}$ of the enzyme without inhibitor as well as the apparent maximum velocities $V_{max}^{app}$ at each [I] were derived from the slopes $\Delta$, as $\Delta = \frac{K_M}{V_{max}}$. Finally, the inhibition constant $K_i$ of the inhibitor for the enzyme was calculated for each [I].

Haemagglutination inhibition assays. Guinea pig red blood cells (gpRBC) were used for the haemagglutination inhibition (HI) assay. The HN inhibitors were assessed in duplicate as reported previously [19]. Compounds were diluted in PBS as a 4X solution for each concentration tested (25 µl/well, 1X final). Each dilution was mixed with 4 haemagglutination units (HAU) of purified hPIV-3 (25 µl/well, 1 HAU final) and incubated for 20 min at room temperature. One haemagglutination unit corresponds to the lowest final concentration of virus that results in a complete agglutination of gpRBC at 4 °C. The plate was transferred on ice and an equivalent volume of ice-cold 1% gpRBC in PBS (50 µl/well)
was added to each well. The plate was incubated for 1 hour and 30 minutes at 4 °C before reading haemagglutination results. The IC$_{50}$ for HI assay was defined as the compound concentration that results in a similar agglutination as the one observed in a control well containing only 0.5 HAU of hPIV-3 and gpRBC.

**Cell-based assays.** The *in vitro*, dose-dependent potency of compounds was determined by immuno-stained focus reduction assay. Confluent cells in 96 or 48-well plates were infected with 20—50 focus forming units (ffu) of hPIV-3 per well for 1 h at 37 °C. The cells were washed with PBS, and overlaid with 100 µL of 0.75% carboxymethylcellulose in un-supplemented DMEM. To test inhibition of virus binding (infectivity), compounds were applied during virus adsorption for 1 h at 4 °C, removed, and the cells were incubated for 72 h at 37 °C. To assess inhibition of virus propagation, compounds were applied after virus adsorption at 37 °C, for 48—72 h at 37 °C. The immuno-staining was performed by fixing cells for 20 min with 4% PFA in PBS at room temperature, followed by a 30 min incubation at 37 °C with a primary anti-hPIV-3 HN monoclonal antibody (clone M02122321, mouse IgG – Fitzgerald, Acton, MA) diluted 1:2000 in PBS-5% skim milk, and a 30 min incubation at 37 °C with a 1:6000 dilution of secondary HRP-conjugated polyclonal antibody (goat anti-mouse IgG (H+L) – Bethyl, Montgomery, TX) in PBS-5% skim milk. Virus-infected cells were stained by addition of TrueBlue Peroxidase Substrate (KPL, Gaithersburg, MD) until appearance of dark blue foci. The concentration of compound resulting in 50% inhibition (IC$_{50}$) of virus binding, or infectivity, was determined by foci counting, while the IC$_{50}$ for virus replication was determined by measurement of foci size. IC$_{50}$ values were determined using non-linear regression analysis with the software GraphPad Prism (GraphPad Software, La Jolla California USA).
Foci counts and size measurements were done using the software Fiji [40]. Briefly, the backgrounds of 1200 dpi colour images from scanned infection plates were subtracted using a rolling ball radius setting of 10 pixels with the sliding paraboloid option. The colour channels were split and the red channels (channel containing the darkest spots) were smoothened before being thresholded to binary images. The number and average size of foci in each well were determined using the particles analyser.

The toxicity of compounds towards LLC-MK2 cells was evaluated using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), following the manufacturer’s instructions in the conditions of the focus reduction assay.

**Cell-based chemical combination assays.** *In vitro* chemical combinations were performed to assess the synergism or the antagonism of suramin in presence of a competitive inhibitor of hPIV-3 HN to block infection. Infections were performed in a focus reduction assay format, in duplicates in 48-well plates with ~30 focus-forming units of hPIV-3 per well, and the compounds were added post-virus adsorption to mimic post-exposure treatment. The experiments were done following the method of Chou et al. [41], using constant, equipotent ratios of compounds according to their respective IC$_{50}$*in vitro*: 0.25-, 0.5-, 1-, 2-, and 4-fold of IC$_{50}$. The synergism, antagonism, or additive effect of each of the combinations was assessed by calculating the combination index value (CI). According to Chou et al. [41,42],

$$CI = \frac{D_1}{D_{1x}} + \frac{D_2}{D_{2x}},$$

where $D_1$ and $D_2$ are the doses of compound 1 and 2 respectively responsible for an effect $x$ in combination, while $D_{1x}$ and $D_{2x}$ are the doses of compound 1 and 2 respectively responsible for the same effect individually. If CI<1 the compounds have a synergistic effect, if CI>1 they are antagonistic, and if CI=1 additive effect is observed. A normalised isobologram was created by plotting the normalised concentrations $\frac{D_1}{D_{1x}}$ of
compound 1 and $\frac{D_1}{D_2}$ of compound 2 on the y- and x-axis respectively; where the
denominators represent the respective doses of compound 1 and compound 2 alone reducing
viral load by $x\%$, and the numerators represent the respective doses of compound 1 and
compound 2 reducing viral load by $x\%$ in combination. The normalised concentrations were
calculated given that $\frac{D_1}{D_1^x} = \frac{D_1}{D_{m1}} \left( \frac{1 - f_a}{f_a} \right)^{m_1}$, where $D_{m1}$ is the IC$_{50}$ of compound 1 in vitro,
$f_a$ is the fraction affected (or (% effect) ÷ 100), and $m_1$ is the slope of linear regressions from
median effect plots where $\log(\frac{f_a}{1 - f_a})$ is plotted as a function of the log of compound
concentration. The CI-Effect graph representing the CI as a function of the associated
antiviral effect was also plotted, as well as the log(DRI)-effect plot representing the log of the
dose reduction index (DRI) as a function of the associated antiviral effect. The DRI is the
ratio of the concentration of a compound resulting in an effect $x$ alone ($D_1^x$), to the
concentration of the same compound resulting in an effect $x$ in combination ($D_1$): $DRI = \frac{D_1^x}{D_1}$.

**PEG-precipitation of hPIV-3 particles.** For neuraminidase inhibition assays (NI) and STD-NMR experiments, hPIV-3 prepared by propagation in LLC-MK2 cells at a MOI of 1 were precipitated by treating clarified virus-containing supernatant of infection with PEG-6000. A 5X solution of sterile 40% PEG-6000 in PBS was slowly added to the supernatants, and the tubes were kept at 4 °C for 2 h under gentle agitation. They were centrifuged for 15 min at 3000 × $g$ at 4 °C, and the supernatants were discarded. The pellets were soaked at 4 °C overnight with 1/100$^{th}$ of the initial volume of GNTE buffer (200 mM glycine, 200 mM NaCl, 20 mM Tris-HCl, 2mM EDTA, pH 7.5). They were resuspended on the next day by gentle
up-and-down pipetting. The precipitated virus was pooled and kept at 4 °C for later use in
neuraminidase assay.

For STD-NMR experiments, the hPIV-3 particles were purified on a 30%-60% non-linear
sucrose gradient in GNTE buffer [43], as previously described [19]. The PEG-precipitated
virus was firstly resuspended in GNTE buffer using a dounce homogeniser, and loaded onto
the gradient. The particles were purified by centrifugation at 100,000 x g for 2 h 30 without
break at 4 ºC, using a SW 32.1 Ti Rotor (Beckman Coulter, Brea, CA).

**Competition STD-NMR experiments.** Purified hPIV-3 was firstly inactivated by exposition
to UV light for 10 min. It was then loaded into a 0.5 mL 30 kD cut-off concentration column
(Millipore, Billerica, MA) and the virus purification buffer was exchanged at least 4 times
with deuterated NaOAc 50 mM, CaCl₂ 5 mM, pH 4.6, following the manufacturer’s
instruction. For the single-compound STD-NMR, the virus was resuspended into exchange
buffer to a final volume of 200 μL and suramin or the competitive neuraminidase inhibitor
zanamivir dissolved in D₂O was added at a final concentration of 5 mM. A control STD-
NMR spectrum was acquired for each compound, in absence of virus. For competition STD-
NMR experiments, the sample was reprocessed after receiving an equimolar concentration of
the other inhibitor. The 1D proton (¹H) NMR and STD-NMR spectra were acquired at 600
MHz and 281 °K, as described previously [19]. For STD-NMR, the on-resonance frequency
was set to -1.00 ppm and the off-resonance frequency to 300 ppm. The virus was saturated
with 60 Gaussian soft pulses of 50 ms, each resulting in a total saturation time of 3 s. The
residual water signal was reduced by applying the WATERGATE sequence.

**Molecular docking simulations.** Simulations were carried out using the software Autodock
Vina [44]. The apo- and zanamivir-bound structures of HN were taken from the Protein Data
Bank, accession numbers 1V3B and 1V3E respectively. Water molecules were removed, as
well as B-chains to keep only one monomer for the simulations. The half-suramin molecule
(suramin\textsuperscript{b}), truncated after the urea moiety (NHCONH\textsubscript{2}-term), was optimised through the
PRODRG2 server \cite{45}. Zanamivir was extracted directly from the crystal structure 1V3E. All
simulations were performed on a 30 × 30 × 30 Å grid box (27,000 Å\textsuperscript{3}). For the blind docking
experiments, 36 grids were designed to overlap by 15 Å in all three x, y and z directions, to
reconstitute a global search space of 60 × 60 × 75 Å (270,000 Å\textsuperscript{3}) containing an entire HN
monomer. Simulations were run on Griffith University High Performance Computing Cluster
"Gowonda" with an exhaustiveness parameter of 16, and 10-50 conformations were
generated for each simulation.
Results

Enzyme-based semi-high throughput screening of hPIV-3 neuraminidase inhibitors.

In our desire to discover novel, repurposable inhibitors of hPIV-3 neuraminidase, we screened a library of 1280 USA and International approved drugs in neuraminidase inhibition assay. The drugs, stored in DMSO, were tested at a final concentration of 100 µM. This concentration was the highest that could be achieved while allowing the efficient dissolution of compounds in an aqueous buffer, with an acceptable DMSO concentration (1% final), and resulting in a less than 20% loss of signal (data not shown). The quality of the assay was determined by calculation of the Z-factor [46]. Throughout the screening we obtained an average Z-factor = 0.74 ± 0.19, indicating that the assay was reliable and within a good dynamic range.

In primary screening, a threshold of 80% reduction of neuraminidase activity was chosen to discriminate compounds with an IC\textsubscript{50} above 100 µM. Out of 1280 drugs, three inhibitors of the hPIV-3 neuraminidase were identified and confirmed in a secondary screening (data not shown): suramin (Figure 2A), evans blue and erythrosine sodium, with neuraminidase inhibitions of 94 ± 0.2%, 79.4 ± 1.8% and 92.8 ± 0.9%, respectively. Evans blue and erythrosine sodium being dyes, their quenching effect on fluorescence was evaluated by adding the compounds at the end of the enzymatic reaction. No decrease in fluorescence was observed, showing no quenching effect of the dyes. However, due to the nature of the compounds, they were not further evaluated as inhibitors of hPIV-3 in this study.
Suramin inhibits the haemagglutinin and neuraminidase activities of hPIV-3 HN in a
dose-dependent manner.

The potency of Suramin as an inhibitor of hPIV-3 neuraminidase was further characterised by
dose-response experiments. The NI assay was carried out following the conditions of the
primary screening, using serial dilutions of drug. Suramin was found to inhibit HN
neuraminidase in a dose-dependent manner, with an IC$_{50}$ of 13.5 µM (Figure 2B). As a
comparison, compound 10 had a reported IC$_{50}$ of 1.5 µM [19] (Table 1).

As the neuraminidase active site of HN is also a sialic acid binding site, we tested whether the
drug could block the haemagglutinin activity of the protein. In this assay, we challenged the
capacity of HN to agglutinate guinea pig red blood cells (gpRBC) in presence of suramin. We
qualitatively observed the agglutination of gpRBC after 1 h 30 min of incubation with the
virus and the drug at 4 °C. The dose required for suramin to inhibit 50% of the
haemagglutinin activity was about 500 µM, against 2.7 µM for compound 10 [19] (Table 1).

Figure 2. Enzymatic inhibition of HN by suramin. (A). Chemical structure of suramin. (B).
Dose-response of suramin against hPIV-3 neuraminidase. Data points are the mean of
duplicate values and are representative of a least 2 independent experiments. The error bars
represent the SEM.
Table 1. Comparison of the activities of suramin and compound 10 as inhibitors of hPIV-3 HN

<table>
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<th>Compound</th>
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<tr>
<td>Suramin</td>
<td>500</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Compound 10 [19]</td>
<td>2.7</td>
<td>1.5</td>
<td></td>
</tr>
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HA: haemagglutinin activity
NA: neuraminidase activity

Suramin has antiviral activity in vitro.

We evaluated the dose-dependent antiviral potency of suramin on hPIV-3-infected LLC-MK2 cells by immuno-stained focus reduction assay. We tested the drug at adsorption stage at 4 °C and 37 °C for an hour to evaluate the effect on virus binding (4 °C) and early events of infection including fusion (37 °C). After 1 h of adsorption, the inoculum was removed and replaced with fresh medium without drug. In another experiment the drug was added post-adsorption to evaluate post-entry effects. The virus was replaced with fresh medium supplemented with suramin after 1 h of virus adsorption at 37 °C. Interestingly, suramin had the most potent antiviral effect during virus adsorption at 4 °C (binding event) with an IC_{50} value of 3.1 µM, showing that the drug efficiently blocked hPIV-3 HN receptor binding site and prevented entry (Figure 3A). Suramin also inhibited infection during adsorption at 37 °C (binding and fusion events) but with a lesser efficacy (IC_{50} = 26 µM). When added post-adsorption, the drug had an IC_{50} value of 35 µM, against 100 µM for zanamivir in the same experimental conditions (Figure 3B). As shown on Figure 3C, the reduction of the average size of foci by suramin could be accurately measured using automated high-resolution image treatments. While the number of foci remained constant, their size could be reduced down to the size of single infected cells.
Figure 3. Antiviral effect in vitro of suramin and zanamivir on hPIV-3-infected LLC-MK2 cells. Dose-dependent inhibition of hPIV-3 infection by suramin at different stages of infection (A), and by suramin and zanamivir post-adsorption (B). The antiviral potencies of the drugs were evaluated by focus reduction assay, and the drugs were added either during virus binding (4 °C for 1 h), at adsorption stage (37 °C for 1 h), or post-adsorption (37 °C for 72 h). The immuno-staining was carried out after 72 h of incubation. (C). Post-adsorption effect of suramin on reduction of foci size at 30 µM as compared to an untreated control (mock). Top: scan of a focus reduction assay from a 24-well plate immuno-stained 72 h post-infection. Bottom: image of the same well after image conversion to binary image and particle detection for automated foci counting and size measurements using Fiji. Each detected focus is outlined in black and numbered in red.
Suramin inhibits the hPIV-3 neuraminidase via a non-competitive mechanism. Although suramin has been shown to inhibit some neuraminidases, its mechanism of action remains unknown [47–49]. To understand the mode of inhibition of the drug on HN, we first conducted enzyme kinetics analysis of the neuraminidase in presence of suramin and the substrate MUN. In the setup of the neuraminidase inhibition experiment, we evaluated the initial velocity \( v_i \) of the neuraminidase at several concentrations of substrate ([S]). At each of these substrate concentrations, we challenged the enzyme with a dilution range of suramin ([I]). Linear regressions of the function \( \frac{1}{v_i} = f\left(\frac{1}{[S]}\right) \) were computed for each concentration of suramin (Figure 4). They all converge and cross the x-axis at a single value. The constant of Michaelis-Menten \( K_M \), which translates the affinity of HN for the substrate, was deduced from this point of convergence and found to be of 29.10 ± 1.45 mM. The constant of inhibition \( K_i \) of suramin was equal to 4.88 ± 0.48 µM. On the contrary, the maximum velocity of the enzyme \( V_{\text{max}} \) was equal to 0.068 mmol/sec, and was reduced when suramin was present in the reaction with apparent maximum velocities \( V_{\text{app max}} \) values of 0.051, 0.04 and 0.019 mmol/sec at drug concentrations of 6, 10 and 16 µM, respectively. These data strongly suggest that suramin acts on the hPIV-3 HN via a non-competitive mechanism, and that it does not bind directly to the protein primary binding site and does not compete with N-acetyleneuraminic acid.
Figure 4. Determination of the inhibition mode of suramin by enzyme kinetics. The initial velocities $v_i$ of the neuraminidase were determined at several concentrations of fluorogenic substrate MUN (2, 4, 8, 16, 20 mM) for each concentration of suramin [suramin]. The Lineweaver-Burke graph was created by plotting duplicate values of $\frac{1}{v_i}$ as a function of $\frac{1}{[S]}$, and is representative of 3 independent experiments. The straight lines are linear regressions calculated for each concentration of inhibitor.

To validate the non-competitive inhibition of the hPIV-3 neuraminidase by suramin, we followed another approach using a competition-STD-NMR experiment in presence of the approved drug zanamivir. We analysed the change in binding of zanamivir to purified hPIV-3 particles upon addition of suramin, as well as the change in binding of suramin upon addition of zanamivir. As expected, STD-NMR signals of both drugs were obtained, thus confirming their binding to HN (Figure 5B-C). Although their $^1$H NMR signals were equivalent in intensities (Figure 5A), the STD-NMR signals of suramin were higher than those of
zanamivir, mainly due to the perfect symmetry of the molecule and to the consequent amplification of the signal of each proton. The sequential order in which the drugs were added to the biological sample did not influence the final STD-NMR spectra of the drug combinations (*Figure 5B versus C*). The STD-NMR intensities of suramin remained unchanged upon addition of zanamivir (*Figure 5D*). The STD-NMR intensities of zanamivir, however, were increased by ~30% upon addition of suramin (*Figure 5E*). Similar intensities were obtained when suramin was added before zanamivir, which suggests that suramin binding to HN modifies the steric environment of the primary binding site, thus influencing zanamivir binding.
Figure 5. Competition STD-NMR of suramin and zanamivir in presence of purified hPIV-3. (A). $^1$H-NMR spectrum of suramin and zanamivir. (B) and (C). Competition STD-NMR spectra of suramin and zanamivir in presence of virus where zanamivir was added before (B) or after (C) suramin. Absolute intensities of (B) and (C) are comparable. (D). STD-NMR spectra of suramin alone in presence of virus (bottom), and after addition of zanamivir (top). (E). STD-NMR spectra of zanamivir alone in presence of virus (bottom), and after addition of suramin (top). Drugs in combination were tested at an equimolar ratio.
Suramin acts in synergy with competitive inhibitors of HN to block infection \textit{in vitro}.

As suramin is a non-competitive inhibitor of HN, it is expected to have a synergistic effect when combined with a competitive inhibitor since both compounds would not bind the same region of the protein. Suramin being an approved drug, we tested this hypothesis with the approved competitive inhibitor zanamivir to see if lower concentrations of both drugs could be used to block infection \textit{in vitro}. We also evaluated combinations with compound 10, a recently designed hPIV-3 HN inhibitor, to observe if a synergy could be observed with better inhibitors. We used the method of Chou and Talalay to design the experiments and assess the combinations \cite{41,50,51}. The drugs were tested at constant ratios of equipotent concentrations, ranging from 0.25 to 4-fold of their respective IC$_{50}$ that was re-determined for each experiment (Figure 6A, Table 2). In this particular assay, suramin had an average IC$_{50}$ value of 74.5 µM, against 61.5 µM for zanamivir and 2.2 µM for compound 10. The slopes $m$ from linear regression of median-effect plots were also determined, as they reflect the sigmoidicity of the dose-response curves and were used for the subsequent calculation of normalised concentrations and drug reduction indexes (Figure 6B). The antiviral effects of individual compounds and compounds in combinations are reported in Table 2.

The normalised isobologram is a graphical way of visualising synergistic combinations with regard to concentrations. Since $D_1$ is the concentration of compound 1 responsible for an effect $x$ in combination, and $D_{1,x}$ the concentration of compound 1 responsible for an effect $x$ on its own, a normalised concentration $\frac{D_1}{D_{1,x}}$ tends to zero as lower concentrations of compounds in combination are required to reach an effect $x$. As shown in Figure 6C, all data points, to the exception of one, are located in region $a$ where combinations have a synergistic effect, suggesting that both zanamivir and compound 10 are acting in synergy with suramin to block hPIV-3 infection. The CI-effect plot also allows the visualisation of combination
effects, based on the combination index CI. It represents the CI value as a function of the associated antiviral effect for each combination, and a CI value <1, >1 and = 1 is representative of synergistic, antagonistic and additive effects, respectively (Figure 6D). Similarly to Figure 6C, all combination points to the exception of one were inferior to 1, indicating a synergistic effect between suramin and zanamivir or compound 10.

The fact that the compounds show synergism means that their concentration in combination yields an effect that is stronger than individual compounds at a similar or higher concentration. This property of synergistic inhibitors can be evaluated through the calculation of the drug reduction index (DRI) for each molecule from each combination, and is represented with the log(DRI)-effect plot (Figure 6E). In the case of suramin—zanamivir or suramin—compound 10 combinations to inhibit hPIV-3 infection, the stronger the effect, the higher the DRI. Although this result is what is expected, it does not translate to high synergism as the DRI is calculated for individual drugs at a given effect in combination. According to the DRI-effect plot, by reducing by a factor 5 the concentrations of compounds individually required to reach 100% of inhibition, the same level of antiviral effect can be obtained in combination. In addition, as it is reported in Table 2 only 2 × IC50 of suramin and zanamivir, and 2 × IC50 suramin and compound 10 used in combination are sufficient to inhibit 100% of hPIV-3 infection in vitro, against 4 × IC50 for any drug used individually.

<table>
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Table 2. Antiviral effect of suramin in combination with zanamivir and compound 10 (Cp. 10).
Figure 6. Evaluation of the synergism of suramin in combination with competitive inhibitors of HN in vitro. Red and blue data points correspond to suramin—compound 10 or suramin—zanamivir combinations, respectively. (A). Dose-response curves of each individual compound. Suramin was evaluated twice, for each of the combinations with suramin and compound 10. (B). Median-effect representation of the dose-response curves for each individual compound. $m$ is the slope of the linear regressions, $f_a$ is the “fraction affected”, or ($\%$ effect) $\div$ 100. (C). Normalised isobologram representing, for each combination, the normalised dose of each compound individually required to reach the observed effect in combination. $D_1$ is the dose of a compound 1 in combination required to achieve $x\%$ of inhibition, while $D_{x1}$ is the dose of a compound alone required to achieve $x\%$
of inhibition. Data points in zone $a$, $b$ and $c$ correspond to combinations with synergistic, additive or antagonistic effects, respectively. (D). CI-effect plot representing the combination index CI of each combination as a function of their associated antiviral effect. The zones $a$, $b$ and $c$ are the same as the ones described in (C). (E). log(DRI)-effect plot representing the drug reduction index (DRI) of compounds as a function of their antiviral effect in combination. The DRI is calculated for each drug in each combination, and represents the dilution factor required for a drug to reach the same level of inhibition individually as in combination. All combinations were tested in quadruplicates, post-adsorption, by focus reduction assay. The results are representative of 3 independent experiments.

A truncated suramin binds in the vicinity of the HN active site in in silico docking simulations.

We used in silico docking experiments to identify a potential binding site for suramin on hPIV-3 HN. Since suramin contains a high number of torsion sites that can impair the efficiency of simulations when using Autodock or Autodock Vina, and since it is a symmetrical molecule, we cleaved it after the median urea group to leave a terminal R-NHCONH moiety with R being the polycyclic half of the molecule. As a positive control for the docking simulations, zanamivir from the crystal structure 1V3E was re-docked into the active site of HN. As shown on Figure 7A, the conformations of best energies overlapped with the crystal structure of zanamivir. The pink and light pink structures were in opposite directions as compared to the zanamivir from the crystal structure, but the magenta structure was positioned very closely to zanamivir with an RMSD = 0.46 Å (RMSD: root mean square deviation).

We designed a blind-docking experiment to probe the binding site of this half-suramin (suramin$^b$) on hPIV-3 HN, by performing simulations with Autodock Vina on 36 overlapping search spaces covering an entire monomer (Figure 7B). A total of 10 conformations were
generated for each of the 36 search spaces. The simulations performed to probe suramin binding site were done on the apo-form of HN (1V3B), as well as on the zanamivir-bound form (IV3E) with zanamivir included in the HN primary binding site. On the apo-structure, suramin$^h$ was positioned with the lowest energy into HN active site (Figure 7C). However, as it is a very bulky molecule and the other half would also need to be accommodated in an in vitro setup, it is very unlikely that suramin$^h$ would actually fit into the active site as shown by the surface representation of the compound. Interestingly, suramin$^h$ was also positioned with the lowest energy around the active site and over zanamivir on the zanamivir-bound structure. The cyan and magenta structures on Figure 7D are the models of lowest energies of simulations run on 2 overlapping search spaces that include regions of the active site. Given the orientation of the docked models of suramin$^h$, the other half of suramin could easily be accommodated in both cases as the urea moieties remain accessible. Suramin$^h$ was however found to have no particular affinity for HN dimeric interface in silico (data not shown).
Figure 7. Probing of suramin binding site on hPIV-3 HN. (A). Solvent-accessible surface representation of HN active site (1V3E, grey), with zanamivir docked in. Zanamivir from the crystal structure 1V3E is represented in orange sticks, the 3 best conformations of zanamivir from docking simulations are represented in magenta, pink and light pink sticks. (B). Suramin\(^b\) binding site probing strategy. A total of 36 overlapping grids (search spaces) were designed to cover the entire surface of an HN monomer (represented in grey), and simulations were run on each of them. Left: volume of the 36 grids combined. Right: volume of a single grid centred on the HN active site. (C) and (D). Solvent-accessible surface representation of HN with the best conformations of suramin\(^b\) docked into the active site of the apo-form (1V3B, suramin: magenta sticks, grey surface) (C), or over the active site of HN bound to zanamivir (1V3E; zanamivir: green sticks, grey surface; suramin\(^b\): cyan and magenta sticks) (D). HN surface in (C) and (D) is coloured from red (-15 kT/e) to blue (15 kT/e) according to its electrostatic potential (APBS).
Our approach in this study was to discover novel scaffolds of neuraminidase inhibitors that could be repositionable drugs, and work either in cooperativity with other inhibitors, or act as a tool to better understand the mechanisms by which the protein functions are regulated. Following a target-based drug discovery strategy, we identified the approved drug suramin as a novel inhibitor of hPIV-3 HN. We found that it acts as a non-competitive inhibitor for the enzyme, and in synergy with competitive inhibitors of HN to block hPIV-3 infection in vitro.

We discovered the anti-hPIV-3 neuraminidase activity of suramin by enzyme-based semi-high throughput screening of approved drugs on the neuraminidase function of HN. This is, to our knowledge, the first of its kind conducted on the parainfluenza neuraminidase. The drug, not related to sialic acid, was found to have activity both on the neuraminidase and the haemagglutinin functions of HN, although with a lower potency as compared the novel benchmark-inhibitor compound 10.

The kinetic analysis we conducted on HN with suramin showed that the drug did not compete with the substrate. While the apparent maximum velocity of the enzyme changed significantly in presence of drug, its constant of affinity for the substrate remained unaffected. The binding of suramin and the MUN substrate to HN are therefore unaffected by one another, meaning that they bind to distinct sites on the protein. To further investigate this property of suramin, we conducted competition STD-NMR experiments with suramin and zanamivir using purified virus particles. Suramin appeared to bind more strongly than zanamivir on HN, judging by the intensities of STD-NMR signals. This can be explained by the fact that suramin being a symmetrical drug, the signals of all proton are enhanced. Also, suramin has a high plasma protein binding potential of 99.7% [52]. Since the experiments were conducted using purified virus particles, it is possible that suramin bound to other viral
proteins in addition to HN. Suramin and zanamivir were both found to bind HN, whether they were in combination or not, confirming the non-competitiveness of suramin towards HN. The STD-NMR signals of zanamivir were found to be enhanced by about 30% upon addition of an equimolar ratio of suramin. Similar STD-NMR signals of zanamivir were found when suramin was added first. This most likely supports the notion that suramin induces a modification in the steric environment of the zanamivir binding site, possibly by rigidifying the 216 flexible loop.

Given that the HN protein possesses a single primary binding domain, suramin would inhibit both entry and release in vitro if it bound closely to the sialic acid recognition site. We found suramin to be the most potent at adsorption stage in vitro, although it is also active post-adsorption. This indicates that suramin prevents the binding of HN to sialic acid-containing receptors, as well as their cleavage. As previously mentioned, suramin has a high protein binding potential. This property of suramin could account to the fact that the drug has better in vitro activity at adsorption stage than post-adsorption. Indeed, as the suramin molecules are bound to the virus and the cells during adsorption stage, they could also stay bound after washing the cells during infection, and suramin could continue to work to block viral replication.

Although it is a non-competitive inhibitor of HN, our results indicate that suramin binds most likely to the vicinity of the active site. The extensive in silico analysis that we conducted in the present study substantiate this hypothesis. We probed the binding site of a truncated variant of suramin by blind docking simulations on 36 overlapping search spaces covering an entire HN monomer. In both the apo-form and the zanamivir-bound forms of HN, the dockings of lowest energies were located around the active site. It appeared that suramin could fit into the active site, however it is very unlikely to happen as the cavity is very small.
with regards to the high molecular weight and flexibility of the drug. Suramin was however shown to bind the vicinity of the active site *in silico* whether zanamivir was bound or not, confirming what was observed in STD-NMR experiments and suggesting that both drugs could act in cooperativity. A crystal structure of hPIV-3 HN in complex with suramin would be of tremendous value to characterise the precise binding mechanism of the drug to the protein.

The sialic acid-based drug zanamivir was shown in several studies to be active against hPIV-3 *in vitro*, although its potency is in the high micromolar range [15,27,53]. This is also the case for suramin, but we showed that the two drugs can act in synergy to inhibit hPIV-3 infection *in vitro*. As a result, we showed that the dose of both drugs can be significantly reduced to reach high levels of inhibition, as compared to the individual dose that would otherwise be needed to reach a similar effect. We also demonstrated that this feature of suramin can be applied to better inhibitors of HN, such as compound 10. Similar levels of synergy as with zanamivir can be achieved in combination, but as it is more potent than zanamivir even lower concentrations of drugs are needed. This data also confirms the non-competitive inhibition of suramin. Indeed, if suramin bound to the active site, either suramin or the competitive inhibitor would occupy the binding pocket and no cooperativity would be observed.

These results are an *in vitro* proof of concept for combinatorial drug repurposing, as both suramin and zanamivir are approved drugs. Suramin, originally a trypanocidal drug, has been found to be a promising clinical candidate for the treatment of enterovirus 71 infection *in vitro and in vivo* [54], showing that a similar approach could be used for the treatment of hPIV-3 infection.
Acknowledgements

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Chapter 3

Phenotypic-based discovery of a potent anti-human respiratory syncytial virus agent

3.1 Preface

Among the various pathogens causing acute respiratory illnesses in infants and elderly, human respiratory syncytial virus certainly is the most prevalent\textsuperscript{1–3}. As presented in the Introduction of this Thesis, the global burden of hRSV-associated disease is socially and economically high. As a consequence of the cost of the prophylactic antibody palivizumab, the health burden in the most affected populations that are in developing countries remains at unacceptable levels\textsuperscript{1,4}. Furthermore, this antibody is only intended to treat at-risk patients\textsuperscript{5,6}. A few drugs have made it through pre-clinical studies and are now being evaluated in clinical trials\textsuperscript{7–9}, but given their small number (3) and the low estimated likelihood of approval of drug candidates from phase I, about 15\%\textsuperscript{10}, global effort towards the discovery of novel drug candidates must be sustained.

The study reported in this Chapter was designed to identify novel inhibitors of hRSV infection, by phenotypic screening of 2 compound libraries containing over 3,700 approved drugs, probe-compounds and natural products. As detailed in Section 1.4.3.1.2 (p. 48), most of the anti-hRSV small molecule drug discovery focusses on fusion protein
inhibitors, and the development of several drug candidates has been halted. The approach in the present study was to obtain novel classes of hRSV inhibitors, that would expend the repertoire of available tools for the development of hRSV drug candidates. Furthermore, phenotypic screening often lead to the identification of unique pathways of host cell infection, when target deconvolution investigations of lead compounds prove successful.

From the CPE-based phenotypic screening that was performed on hRSV infection \textit{in vitro}, two natural products were identified: cyclopamine and jervine. They are potent inhibitors of the Sonic hedgehog signalling pathway (Shhp), that is involved mainly in cell differentiation and embryo development\textsuperscript{11}. They are \textit{Veratrum} alkaloids, isolated from plants of the \textit{Veratrum} genus such as the \textit{Veratrum californicum}, or corn lily. They were originally discovered in the 1950s, when it was found that sheep herds that had ingested the plant gave birth to calves with severe birth defects\textsuperscript{12}. Since then the compounds, especially cyclopamine, have been extensively used to study the regulation of Shhp. An abnormal activation of the Shhp has been observed in various cancers, and a potent inhibitor of Shhp, vismodegib (Erivedge\textsuperscript{®}, Genentech, Inc.) has been approved for the treatment of basal-cell carcinoma\textsuperscript{13–16}.

The Shhp is activated by binding of extracellular hedgehog (Hh) ligand-proteins, Indian, Desert or Sonic hedgehog, or Ihh, Dhh and Shh respectively, to the transmembrane Patched 1 (Ptch) receptor. In the absence of Hh ligands, Ptch normally prevents the translocation of the 7-pass transmembrane receptor Smoothened (Smo) from the cytoplasm to the cell surface, thus inhibiting it. In presence of Hh ligands Ptch is inhibited, which restores the translocation and activation of Smo. This results in a regulation cascade that leads to the activation of the transcription of downstream hedgehog target genes\textsuperscript{17}. The Shhp down-regulators cyclopamine and jervine are potent nanomolar inhibitors and binding partners of Smo, cyclopamine being more potent than jervine\textsuperscript{18,19}.

Subsequently its discovery by phenotypic screening in the present study, cyclopamine (CPM) was further evaluated for its capacity to inhibit hRSV infection \textit{in vitro}, in time-of-addition experiments. CPM being a known inhibitor of Smo and the Shhp, several
tool-compounds, inhibitors and activators of Shhp as well as antibodies, were used in order to determine whether the Shhp was involved in infection. As none of them had any effect on infection, it was concluded that CPM inhibition was due to an off-target effect. A target-deconvolution strategy via resistance selection was therefore chosen so that a viral partner linked to CPM inhibition could be identified. The resistance selection was performed by sequential blind-passage experiments with increasing concentration of compound, and several resistant hRSV strains were obtained. Their genomes were sequenced and compared to a reference strain, which led to the identification of unique mutations on hRSV M2-1 protein. This protein is an anti-terminator of transcription (see Section 1.2.9, p. 21), and is part of the replication complex of the virus. To assess the effect of CPM on the hRSV replication complex, the compound was tested in a minigenome system. In the minigenome system setup, cells are transfected with plasmids encoding the various elements of the complex (P, N, L, M2-1), and the expression of a fully-functional replication complex allows the transcription of a detectable luciferase gene borne by a sub-genomic replicon plasmid. CPM was found to potently inhibit M2-1-dependent transcription in this system, while inducing a decrease in cytoplasmic levels of M2-1. Finally, CPM was evaluated for its efficacy in the mouse model of infection.

Given the history and potential toxicity of CPM, it will require significant additional investigation to see if CPM is indeed a potential drug candidate for hRSV infections, particularly in paediatric infections. It is, however, the first reported nanomolar inhibitor of hRSV M2-1-dependent replication complex, which opens a new space in the discovery of anti-hRSV compounds.

References


3.2 Bailly et al., 2014

Statement of Contribution to Co-Authored Published Paper

This Section includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:


My contribution to the paper involved:

1. The conception and design of the study.
2. The acquisition and interpretation of most data.
   - Generation of drug resistant viruses was performed by Liu, W. and Wang, Y.
   - M2-1 protein levels analysis were performed by Richard, C.A., Galloux, M., Eléouët, J.F.
   - In vivo studies were conducted by Sharma, G., Johansen, L., Pendharkar, V., Sharma, D.C.
3. The preparation of all figures.
4. The preparation of the draft manuscript.

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Benjamin Bailly

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Corresponding Author
Prof. Ralf Altmeyer

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Supervisor
Prof. Mark von Itzstein
Alkaloids cyclopamine and jervine target the hRSV transcription processivity factor M2-1 and inhibit viral replication in vitro and in vivo

Short title: M2-1 is a novel target for hRSV therapy

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Abstract

Human respiratory syncytial virus (hRSV) is a leading cause of acute lower respiratory tract infection in infants, the elderly and immunocompromised individuals. The *Veratrum* steroidal alkaloids cyclopamine and jervine are potent antagonists of the G protein-coupled receptor Smoothened (Smo), which is required for Hedgehog (Hh) signalling in embryonic development and adult stem cell function. We identified cyclopamine (deoxy-jervine) and jervine as potent and selective inhibitors of hRSV replication in vitro and in vivo. Surprisingly, cyclopamine’s antiviral activity (IC₅₀ 36 nM) was not mediated through the Smo receptor but through the viral replication processivity factor M2-1. Sequence analysis of cyclopamine-resistant mutants led to
the observation that a single point mutation in the hRSV genome, corresponding to a R151K mutation in the M2-1 protein, was able to confer resistance to the compound. Using a minigenome assay that exclusively expresses viral proteins composing the hRSV replication complex (L, N, P and M2-1), we confirmed that cyclopamine represses hRSV polymerase activity when using M2-1\textsuperscript{WT} but not the M2-1\textsuperscript{R151K} mutant, and that cyclopamine selectively reduces expression levels of M2-1\textsuperscript{WT}. When treated with cyclopamine, lung titres of hRSV could be reduced in an experimental mouse model of infection. Cyclopamine and cyclopamine analogues are known bioactive molecules in development for cancer therapy. Here we describe a novel off-target effect of cyclopamine that opens a new avenue for therapy of viral infections.
Introduction

Bronchiolitis is a severe lower-respiratory tract infectious disease primarily caused by members of the *Paramyxoviridae* family. Human respiratory syncytial virus (hRSV) is the principal cause of morbidity in children less than 2-years old [1,2] as well as the elderly, immuno-compromised and transplant patients [3–9]. There is no vaccine available to prevent hRSV infection, but the immuno-prophylactic antibody palivizumab [10] is approved for high-risk patients such as premature babies and infants suffering from underlying diseases [10,11]. The broad-spectrum small molecule antiviral ribavirin is available to treat infection, but it has considerable side-effects and limited efficacy [12,13]. A number of drug candidates targeting entry [14,15] and replication steps [16] of the virus life cycle are currently in development.

The viral replication and transcription of hRSV are mediated by the RNA-dependent RNA polymerase (RdRp), composed of 3 major viral proteins: the nucleocapsids protein N, the phosphoprotein P and the large polymerase L [17]. In this complex, the phosphoprotein, an essential co-factor of the polymerase, binds to L and N, which in turn bind to viral RNA (vRNA) [18–20]. Two co-factors, M2-1 and M2-2, are needed for the RdRp to process RNA efficiently. M2-1 is a tetrameric transcription processivity factor that binds to RNA and P via its core domain in a competitive manner [18,19,21]. M2-1 functions as an anti-terminator of transcription, that prevents premature termination of transcription both intra- and inter-genetically [17,22].

Cyclopamine is a well-known antagonist of the smoothened protein (SMO) receptor, a 7-pass transmembrane receptor of the Sonic hedgehog signalling pathway (ShhP) [23,24]. The ShhP is involved in embryonic development, cell differentiation and tumorigenesis [25,26]. The discovery of the cyclopamine anti-proliferative activity has led to the development of novel, cyclopamine-competitive, Smo antagonists for cancer therapy including GDC-0449 (Vismodegib).
and LY2940680. Vismodegib is currently prescribed for the treatment of basal-cell carcinoma [27].

By screening libraries of known bioactive compounds, we identified cyclopamine and jervine as highly potent and selective inhibitors of hRSV replication in vitro. Unexpectedly, the anti-viral effect of cyclopamine was not mediated by the cognate SMO-receptor, but by the hRSV viral processivity factor M2-1. Cyclopamine-induced, M2-1-linked suppression of hRSV transcription in vitro successfully translated to reduction of hRSV lung titres in cyclopamine-treated mice. This novel off-target effect of cyclopamine opens a new avenue for the development of therapies of hRSV infection, using cyclopamine analogues to target the hRSV replication complex.
Materials and Methods

Cells and viruses. Human respiratory syncytial virus Long strain (ATCC reference VR-26) as well as HEp-2 cells (ATCC reference CCL-23) were obtained from Rong Chen (Structural Virology Unit, Institut Pasteur Shanghai). The cells were maintained in DMEM, supplemented with penicillin/streptomycin and 10% FBS, and kept in a humid atmosphere of 5% CO₂ at 37 °C. The virus was passaged in HEp-2 cells in the same conditions, but with medium supplemented with penicillin/streptomycin and 2% FBS. Virus stocks were prepared by infecting confluent HEp-2 cells at a low multiplicity of infection of 0.1 for two to three days until about 30% cytopathic effects (CPE) could be observed. The cells together with the infection medium were then subject to a single freeze/thaw cycle at -80°C to release the bound virus, and the medium was clarified by centrifugation at 2000 × g for 10 min at 4 °C. The supernatant was gently homogenised, aliquoted and stored at -20 °C for later use.

Compounds. Cyclopamine was purchased from Logan Natural Products (Plano, TX), GDC-0449 from Selleck Chemicals (Shanghai, P.R. China), LY2940680 from Biochempartner (Shanghai, P.R. China), and tomatidine from Yingxuan Pharmaceutical (Shanghai, P.R. China). The Veratrum alkaloids were part of the Screen-Well® Natural Product Library from Enzo Life Sciences (Exeter, UK)

Virus titration assay. Confluent HEp-2 cells in 24-well plates were incubated with serial dilutions of virus for 1.5 h at 37 °C to allow for virus adsorption, and washed twice with PBS. They were overlaid with 0.75% carboxymethyl cellulose in DMEM supplemented with antibiotics and 2% FBS, and incubated for 72 h in the conditions described previously. The infection medium was discarded and the cells were washed 3 x 5 min with PBS before being
fixed for 30 min with 4% PFA in PBS at room temperature. The cells were washed as previously, and incubated for 1 h at room temperature with a primary anti-hRSV F antibody (mouse – Fitzgerald, Acton, MA) in PBS-5% skim milk. After another washing step with PBS-0.02% Tween 20, the cells were incubated with a secondary goat anti-mouse HRP-conjugated antibody (Bethyl, Montgomery, TX) in PBS-5% skim milk for 1 h at room temperature. The cells were washed with PBS-0.02% Tween 20 and overlaid with TrueBlue Peroxidase Substrate (KPL, Gaithersburg, MD) until blue foci appeared. The wells were then rinsed gently under running water, dried and the plates were scanned using a conventional scanner for foci counting and size measurement.

**Immuno-stained focus reduction assays.** To test the anti-hRSV potency of compounds, focus reduction assays were performed in a 24- or 96-well plate format. Infections and immuno-stainings were performed as described above, with 50 to 100 focus forming units per well. Compounds were tested either during virus adsorption for 1 h at 4 °C to investigate viral entry, post-adsorption for 72 h at 37 °C to investigate viral propagation and replication, or at all stages of infection. To assess the cytotoxicity of compounds, a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) was carried out in white opaque plates, following the manufacturer’s instruction in the conditions of the focus reduction assay.

**Generation of cyclopamine-resistant hRSV.** Cyclopamine-resistant viruses were generated by sequential blind-passages with increasing concentration of compound at each passage. Infections were carried out in 12-well plates, on confluent cells, at an MOI of 0.1 for the first infection. The plates were frozen at -80 °C when about 50% CPE was observed after 72 h, and the infection medium was clarified by centrifugation at 2000 × g for 5 min at 4 °C. A volume of 100 μL of clarified supernatant was used to infect cells during the next round. The blind passages were
started with 100 nM of cyclopamine, a concentration that was doubled at each passage up to 400 nM, and increased by 200 nM for the subsequent passages to reach a final concentration of 1 μM. When less than 50% CPE could be observed after 72 h, another passage at the same concentration of compound was performed until signs of obvious CPE. Three separate mutants were generated, alongside a wild-type strain that was treated with DMSO only. Each of the 4 strains were amplified, titrated, and stocks were prepared and stored at -20 °C for later use.

**Genome extraction, amplification and sequencing.** The vRNA of each mutant, including the passaged wild-type virus, was extracted from supernatants of infection using a TIANamp Virus RNA extraction Kit (Tiangen Biotech, Beijing) following the manufacturer’s instructions. The genomes were amplified using a SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA). The fragments were checked by agarose gel electrophoresis and retrieved by gel extraction using a TIANgel Midi Purification Kit (Tiangen Biotech, Beijing), following the manufacturer’s instructions. An aliquot of each fragment was sequenced (Sangon Biotech, Shanghai), and the sequences were analysed using the software Lasergene SeqMan Pro v.7.1 (DNASTAR, Madison, WI).

**Minigenome assays and recombinant proteins.** The minigenome assays were performed in triplicates, as described previously [18,19]. Briefly, BSRT7/5 cells stably expressing the T7 RNA polymerase were transfected in 48-well plates for 24 h in the presence of Lipofectamine 2000 (Invitrogen) and 125 ng of pN, 62.5 ng of pL, 37.5 ng of pM2-1 and 125 ng of pP plasmids encoding for N, L, M2-1WT or M2-1R151K, and P hRSV proteins respectively, under control of the T7 promoter. They were co-transfected with 125 ng of a pM/Luc subgenomic bicistronic minireplicon expressing the firefly luciferase gene located downstream of a hRSV M/SH gene junction, as well as 37.5 ng of a pβ-Gal plasmid coding for the β-galactosidase as a reference.
gene. Compound dilutions or DMSO were added during the initial stage of transfection, and left to incubate with the cells. After 24 h of transfection, cells were lysed in luciferase lysis buffer (30 mM Tris pH 7.9, 10 mM MgCl₂, 1 mM DTT, 1% Triton X-1000, 15% glycerol). Luciferase and β-Gal activities were measured for each well as previously described [19] using a Tecan Infinite 200 PRO (Tecan Group LTD, Männedorf). Recombinant GST-M2-1 and GST-P fusion proteins were obtained as described previously [18].

Electrophoretic motility shift assays (EMSA) and GST pulldowns. M2-1 binding to RNA and P was investigated by EMSA and GST pull down respectively, following the methods previously described by Blondot et al. [18]. A volume of 10 μL of glutathione-eluted GST-M2-1 from glutathione-Sepharose beads (100 μM final) was incubated with 1 μL of cyclopamine dilution (1% DMSO final) and 1 μL of yeast tRNA (Sigma-Aldricht, 5 μM final) for 30 min at room temperature. Complexes were resolved by agarose gel electrophoresis and stained with ethidium bromide. Pulldown of M2-1 by P was achieved by incubating 10 μL of thrombin-cleaved M2-1 with 1 μL of cyclopamine dilution (10% DMSO final) for 15 min at room temperature, followed by 30 min incubation with 50 μL of GST-P on Sepharose beads at room temperature. The beads were washed extensively with PBS and the complexes were resolved by coomassie blue-stained SDS-PAGE.

Immuno-fluorescence microscopy. BSRT7/5 cells transfected with the minigenome and treated with compound or DMSO in 48-well plates during 24 h were fixed for 20 min in 4% PFA in PBS and permeabilised for 5 min in 0.1% triton X-100 and 3% BSA in PBS. The cells were incubated with a primary rabbit polyclonal anti-N antibody [28] and a mouse monoclonal anti-M2-1 antibody (22K4) [29] in blocking solution (0.05% Tween 20 and 3% BSA in PBS) for 1 h, washed, and incubated with secondary goat anti-mouse Alexa Fluor 488 and goat anti-rabbit
Alexa Fluor 633 antibodies in blocking solution. The cells were observed with a Nikon TE200 microscope equipped with a CoolSNAP ES2 (Photometrics) camera, and images were processed using the Meta-Vue (Molecular Devices) and Fiji [30] softwares.

**Immunoassays.** Proteins from SDS-PAGE were transferred onto nitrocellulose membranes. The membranes were incubated in blocking solution for 1 h. Blots were rinsed with PBS containing 0.1% Tween 20 and incubated overnight in blocking solution with primary antibodies: rabbit polyclonal M2-1 [28] and mouse monoclonal tubulin-α antibodies (Sigma-Aldrich, Saint-Quentin Fallavier, France). The membranes were rinsed as described above and incubated for 1 h with the appropriate HRP-conjugated secondary antibodies diluted in blocking solution. The membranes were rinsed, and immunodetections were performed by using an enhanced chemiluminescence (ECL) substrate (GE Healthcare GMB, Vélizy, France) and CL-XPosure films (Thermo Scientific).

**In vivo experiments in the mouse model.** The *in vivo* antiviral efficacy of cyclopamine (CPM) in the BALB/c mouse host model of RSV infection was examined. Female BALB/c mice were procured from Biological Resource Center, Singapore, and used at a body weight of 16 to 20 g. All animals were housed in a certified, specific pathogen-free facility and were fed and watered *ad libitum*. Protocols for use of animals were approved by the Biological Resource Center, Singapore Animal Use and Care Committee.

CPM was formulated as a 0.5% Hydroxypropyl methyl cellulose suspension and was administered at a dose of 30 and 100 mg/kg intraperitoneally bid for four days. BMS-433771, an RSV fusion inhibitor used as a comparator was dissolved in sterile water, and the solution was adjusted to pH 2 to 3.5 with HCl (0.1 N) and was administered at a dose of 50 mg/kg by oral
gavage. Compound treatments were initiated 1 h prior to RSV inoculation. For virus infection,
mice were anesthetised by an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (20 mg/kg) and inoculated by the intranasal route, drop-wise, with $8 \times 10^5$ TCID$_{50}$ of RSV Long Strain in a 100 μl cell culture medium. The control group animals did not receive any treatment.

On day 4 after RSV inoculation, all test animals were euthanised by CO$_2$ gas asphyxiation, and the lungs were excised, weighed, and prepared as homogenates for viral titration. Lungs were homogenised (10%, wt/vol) in a Hanks balanced salt solution containing 0.21 M sucrose, 25 mM HEPES, and 5 mM sodium l-glutamate, supplemented with 20 U/ml of penicillin G, 20 μg/ml of streptomycin, and 0.05 μg/ml of amphotericin B (GIBCO/BRL, Carlsbad, Calif.). Lung homogenates were frozen on dry ice, thawed to release cell-associated virus, and then held on ice until clarification by centrifugation at 300 × g for 10 min at 4 °C. The resulting supernatant samples were immediately titrated for RSV infectivity in HEp-2 cells as described previously [31]. Final RSV lung titres for each animal were calculated as the reciprocal of the log$_{10}$ dilution of TCID$_{50}$ and were expressed as log$_{10}$ TCID$_{50}$ per gram of lung. Infectious RSV lung titres for the animal treatment groups, or cohorts, were calculated as the geometric mean titres and also expressed as log$_{10}$ TCID$_{50}$ per gram of lung. The lower limit of infectious RSV detection for this assay was $\sim 2.8$ log$_{10}$ TCID$_{50}$ per gram of lung. All groups had n=6.
Results

Identification of *Veratrum* alkaloids as inhibitors of hRSV infection *in vitro*.

To identify new antiviral compounds against hRSV, phenotypic screens designed to measure the antiviral effect of compounds on hRSV (Long strain) replication in HEp-2 cells were performed. The screening of a natural product library composed of 502 compounds and an of an approved drug and probe-compound library composed of 3,244 compounds allowed us to identify jervine and cyclopamine (CPM), two members of the *Veratrum* alkaloids family, as potent anti-hRSV molecules *in vitro*. Other compounds of this alkaloids family such as veratrine, portoveratrine-B, imperialine or veratramine, did not present anti-hRSV potency, suggesting a specificity of action of jervine and CPM (Figure 1A).

A focus reduction assay was then developed to further characterise the 50%, 90%, 95% and 99% inhibitory concentrations of the compounds (IC$_{50}$, IC$_{90}$, IC$_{95}$ and IC$_{99}$, respectively). CPM, also known as 11-deoxy jervine, was identified as a very potent inhibitor of hRSV infection with an IC$_{50}$ of 36 nM, IC$_{90}$ of 151 nM, IC$_{99}$ of 336 nM and a selectivity index >2000 when tested during all stages of infection. A simple time of addition assay was performed to identify the stage of the viral life cycle the most likely affected by CPM. When CPM was added exclusively post-adsorption, we observed a 4.4-fold increase in IC$_{50}$ (adsorption vs. post-adsorption; 516 nM vs. 116 nM) and a 10.4-fold increase in IC$_{95}$ (adsorption vs. post-adsorption; 5.4 µM vs. 404 nM) (Figure 1B). It is noteworthy that CPM was not active against another parainfluenza virus, the human parainfluenza type-3 virus (data not shown). Jervine also inhibited hRSV replication at post-adsorption stage with IC$_{50}$ and IC$_{95}$ values of 994 nM and 6.9 µM, respectively, but is less potent than CPM (Figure 1C).
Altogether, these results suggest that CPM and Jervine likely act on a post-entry mechanism of hRSV replication. Due to the greater potency and wealth of available literature data on its biological activity, CPM was then used for all further studies.

Figure 1. Inhibition of hRSV infection by cyclopamine and jervine in vitro. (A). Single dose inhibition of hRSV infection by analogues of cyclopamine tested at 3.5 μg/mL (4—9 μM). (B). Dose-response and cytotoxicity of cyclopamine and jervine against hRSV infection at post-adsorption stage. (C) Dose-response of cyclopamine against hRSV infection at viral adsorption stage, post-adsorption and all stages of infection. Antiviral activities were measured by focus reduction assay. Foci numbers (adsorption stage) and sizes (post-adsorption) were measured automatically using the software FiJi [30], and were normalised as percentage of the untreated control. Cytotoxicity of compounds was measured over 72 h of incubation with healthy cells. Each data point represents the mean of duplicate values, and the SEM is represented by the error bars. The graphs and the IC\textsubscript{50} and IC\textsubscript{95} values were created and calculated using the software GraphPad Prism v.5 (GraphPad Software, La Jolla California, USA), and are representative of 3 independent experiments.
CPM inhibits hRSV infection independently of the smoothened receptor (SMO).

To assess whether Smo is involved in hRSV replication, two inhibitors (GDC-0449, LY2940680) and one activator (purmorphamine) of the pathway, as well as an inactive structural analogue of CPM were tested on hRSV infection in vitro. As shown in Figure 2A, all compounds failed to inhibit hRSV replication at concentrations up to 30 µM, while 1 µM of CPM was sufficient to inhibit 100% of replication. Since GDC-0449, LY2940680 and purmorphamine are known to compete with CPM for binding to SMO [33–35], we performed a competition assay with increasing concentrations of CPM in presence of the compounds. None of the three CPM competitors induced a shift in CPM IC₅₀ for hRSV inhibition compared to CPM alone (Figure 2B).

These results suggest that the inhibition of hRSV infection upon CPM treatment is not mediated by its binding to SMO and that its mechanism of action is independent of the Shh pathway.
Figure 2. Effect of Smo modulators and a CPM analog on hRSV infection in vitro. (A). Dose-response of Smo inhibitors (GDC-0449, LY2940680), a Smo activator (Purmorphamine) and a CPM structural analog (Tomatidine) (left), versus the effect of 1 µM of cyclopamine (right) on hRSV infection in vitro. (B). Dose-response of cyclopamine in combination with 1 µM of GDC-0449 or LY294068 (left); and 5 µM of purmorphamine (right) against hRSV infection. Antiviral activity was measured by focus reduction assay, post-adsorption. The size of foci were measured automatically using the software Fiji [30], and normalised as percentage of the untreated control. Each data point represents the mean of duplicate values, and the SEM is represented by the error bars. The graphs were created using the software GraphPad Prism v.5 (GraphPad Software, La Jolla California USA), and are representative of at least 2 independent experiments.
The R151K mutation in M2-1 confers resistance to CPM.

To determine whether a particular viral protein was implicated in the inhibition of hRSV by CPM, we sought to generate CPM-resistant mutants of hRSV by a resistance selection experiment. We cultured hRSV for eight consecutive passages in the presence of increasing CPM concentrations, starting from 100 nM (WT IC$_{50} = 116$ nM) up to 1 μM (WT IC$_{95} = 404$ nM). Three cyclopamine-resistant hRSV culture supernatants were generated independently, CPM$^R$-1, CPM$^R$-2 and CPM$^R$-3. The resistant viruses had growth characteristics comparable to a wild-type virus passaged in parallel (WTp), and displayed similar focus morphologies in the absence of CPM (Figure 3A).

The potency of cyclopamine, added post-adsorption, was evaluated for each of the CPM-resistant supernatants. As shown in Figure 3B, the WTp supernatant was CPM-sensitive with an IC$_{50}$ value of 119 nM, comparable to the original WT hRSV. However, virus in CPM$^R$-1, CPM$^R$-2 and CPM$^R$-3 supernatants showed a 38—96-fold increase of IC$_{50}$, with values of 6 μM, 4.5 μM and 11.22 μM for CPM$^R$-1, CPM$^R$-2 and CPM$^R$-3, respectively, indicating the presence of resistant virus in the sample.

In order to identify some mutation(s) in the hRSV genome that could be associated with CPM resistance, the entire genome of the four viruses was sequenced. WTp, passaged in the absence of CPM, was found to be identical to the original hRSV Long strain sequence. Alignment of the full genome sequences of CPM$^R$-1, CPM$^R$-2 and CPM$^R$-3 and WTp revealed three coding mutations, all of which were located in a single coding sequence of the viral genome, the hRSV M2-1 transcription processivity factor gene (Figure 3C). Virus in CPM$^R$-1 consisted of a mixed population between wild-type and mutant M2-1 at the amino-acid Y134H and R151K positions.
CPM-2 consisted of a mixed population between wild-type and mutant M2-1 at both the Q144L and R151K positions. In contrast, CPM-3 was a homogeneous population and displayed only the R151K mutation in M2-1. Sequence alignment of 312 M2-1 sequences published in GenBank showed that Y134, Q144 and R151 are fully conserved among hRSV wild type strains (data not shown). Interestingly, the CPM-3 virus showed the highest resistance to CPM as measured by a shift of the IC$_{50}$. Although the three mutated residues are located within the core domain of M2-1, R151 is specifically located in the $\alpha$-helix $\alpha$8, a region previously described as RNA and P binding domain [18,19] (Figure 3D). Altogether, these data suggest that the conserved R151K mutation is sufficient to confer resistance to CPM.
Chapter 3. Inhibitor of hRSV Infection
Figure 3. hRSV mutants resistant to cyclopamine. (A) Comparison of the formation of foci between the CPM-resistant viruses CPMR-1, CPMR-2, CPMR-3 in presence of cyclopamine, as compared to a passaged-wild-type virus (WTp). (B). Dose-response of cyclopamine on WTp and the 3 resistant mutants, measured by focus reduction assay. The compound was applied post-adsorption. The size of foci, measured using the software Fiji [11], were normalised as percentage of the untreated control. The mean values of conditions tested in duplicates are plotted, along with the SEM represented by the error bars. The results are representative of 3 independent experiments. (C). Sequencing chromatograms of WTp and the 3 resistant mutants, focused on the mutated codons responsible for the amino-acid substitutions. The black arrows indicate the sites of clear mutation. (D). Mapping of the three mutation sites on a monomeric M2-1. Left: Surface representation of a full-length M2-1. Right: Surface (top) and cartoon (bottom) representations of the C-terminal side of M2-1 core domain. The RNA and P binding sites described by Blondot et al. [18] are coloured in red and blue respectively. The amino-acids that belong to both binding sites are coloured in magenta. The three sites of mutation are indicated with black arrows. The models (PDB accession code 4C3B) were rendered using the software PyMOL v.1.7.1.0 (Schrödinger, LLC).

Cyclopamine inhibits M2-1-dependent viral transcription.

To validate the results indicating that some M2-1 mutations confer hRSV resistance to cyclopamine treatment, we used a hRSV-specific minigenome assay. The effect of cyclopamine on viral transcription was analysed in BSRT7/5 cells co-transfected with expression plasmids coding for hRSV N, L, P, M2-1WT or M2-1R151K proteins, and a pM/Luc subgenomic replicon coding for the firefly luciferase which expression depends on a functional hRSV replication/transcription complex. CPM inhibited hRSV polymerase activity, while tomatidine and GDC-0449 did not significantly alter the function of the RdRp complex (Figure 4A). CPM inhibited hRSV polymerase activity in a dose-dependent manner with an IC50 of 380 nM.
(Figure 4B). However, cells expressing the M2-1<sup>R151K</sup> mutant were resistant to CPM inhibition with a maximum inhibition of 26% at 1 µM, which could not be significantly increased by raising the CPM concentration (Figure 4C). This result suggests that CPM blocks the polymerase activity by specifically inhibiting M2-1-mediated hRSV transcription.

**Figure 4. Cyclopamine inhibition of hRSV M2-1-dependent transcription.** Inhibition of hRSV polymerase activity by 8.3 µM of cyclopamine, GDC-0449, tomatidine (A) and dilutions of cyclopamine (B) measured by minigenome assay. (C). Minigenome assay with a WT or a R151K mutant M2-1. Viral RNA synthesis was quantified by measuring the luciferase activity after cell lysis 24 h after transfection. The error bars represent the standard error of the mean of luciferase activity measured in triplicates, normalised against β-Gal activity and expressed as percentage of the control treated with DMSO. The results are representative of 2 independent experiments.
Cyclopamine-induced reduction of M2-1 expression levels.

We then looked into the mechanism by which CPM acts on M2-1 activity. We performed immuno-fluorescence (IF) microscopy on minigenome-transfected BSRT7/5 cells to determine if CPM had an effect on the formation of cytoplasmic inclusion bodies. As shown on Figure 5A, N colocalised with M2-1 in inclusion bodies in the absence and in the presence of cyclopamine, even at a concentration inducing >95% reduction of hRSV replication in a minigenome assay. The expression levels of M2-1\(^{WT}\) and M2-1\(^{R151K}\) in CPM-treated or -untreated minigenome-transfected cells were analysed by Western blotting. As shown in Figure 5B, both the slowest and fastest migrating bands of M2-1\(^{WT}\), which correspond to phosphorylated and unphosphorylated forms of M2-1 respectively, were affected by CPM treatment in a dose-dependent manner. However, when the M2-1\(^{R151K}\) mutant was used in place of M2-1\(^{WT}\), the relative intensity of the two bands of M2-1 remained unaffected. To better characterise the change in M2-1 protein levels upon CPM treatment, we analysed the bands from Western blots using a Gnome system. As shown on Figure 5C, the levels of M2-1\(^{WT}\) were dropped in a dose-dependent manner upon treatment with CPM, independently of the phosphorylation state of the protein. The levels of M2-1\(^{R151K}\), on the other hand, did not vary significantly when subject to CPM treatment; although they were generally lower than the ones of M2-1\(^{WT}\).

Since M2-1 is known to bind to RNA and P, we used EMSA and GST-pulldown assays to investigate if the RNA- or P-binding to M2-1 was affected by the presence of CPM. Figure 5D shows that even a high excess of CPM (100 µM) did not prevent the formation of tRNA—M2-1 complexes in EMSA, demonstrated by the lack of free tRNA. Moreover, the M2-1—P interaction was not affected by the presence of CPM in a GST-pulldown assay (Figure 5E).
These results indicate that the mechanism by which CPM inhibits hRSV RdRp is independent of M2-1 binding to RNA or P.

Altogether, these data show that treatment of cells with CPM affects M2-1 protein levels, and the single R151K mutation prevents this effect.
Figure 5. Effect of cyclopamine on M2-1 expression and complex formation with P and RNA. (A). Immuno-fluorescence (IF) of N and M2-1 in minigenome-transfected BSRT7/5 cells. Cells were treated during transfection with DMSO for control, 1 μM or 20 μM of cyclopamine. N (green) and M2-1 (red) were visualised using a rabbit polyclonal anti-N primary antibody and a monoclonal mouse anti-M2-1 primary antibody, respectively. The white arrows point to the location of cytoplasmic inclusion bodies. Merged images were created using the software Fiji. (B) and (C). Cytoplasmic M2-1 levels from minigenome-transfected cells with indicated concentration of cyclopamine, using M2-1WT or M2-1R151K, resolved by SDS-PAGE and stained by western-blot (B) using polyclonal anti-M2-1 and monoclonal anti-tubulin-α antibodies. Levels of tubulin-α are shown as a control of protein expression and DMSO served as control. The results are representative of 3 independent experiments (C). Associated levels of phosphorylated (M2-1P) and unphosphorylated (M2-1un-P) M2-1WT or M2-1R151K, normalised to levels of tubulin-α and expressed as a percentage of M2-1WT without cyclopamine. Proteins levels were measured from western blots as described in the Methods section. Bars are the mean of 3 independent experiments ± SEM. (D). Electrophoretic motility shift assay (EMSA) of GST-M2-1 eluted from GSH-Sepharose beads in complex with tRNA. Complexes were analysed by gel electrophoresis. (D). GST pulldown of M2-1 by P. The GST-P:M2-1 complexes were resolved by SDS-PAGE stained by coomassie-blue.

Antiviral efficacy of cyclopamine in a mouse model of hRSV infection.

CPM exhibited efficacy against RSV in the BALB/c mouse host model of infection (Figure 6) when administered for four days post inoculation. There was a statistically significant decrease in the lung viral titter of animals treated with CPM when compared to control group (p>0.001). The efficacy was comparable to that observed with BMS-477331. The magnitude of RSV inhibition in the mice was dose dependent, and all the lung viral titres were above the detection limit for this assay, i.e., ~2.85 log10 TCID50 per gram.
Figure 6. Efficacy of cyclopamine against RSV in the mouse BALB/c host model of infection. Animals were inoculated intranasally with RSV Long strain. The compound (30 & 100 mg/kg) was administered intraperitoneally as a 4-day b.i.d. regimen in which the first dose was given 1 h before virus inoculation. Treatment cohorts are shown on the abscissa; animals of the infection control group were virus inoculated but not treated. The infectious RSV lung titres are shown on the ordinate as log_{10} TCID_{50} per gram of lung. Each data point symbol represents the RSV titre for each individual animal of the respective treatment cohort. The horizontal line, drawn in each cohort, marks the geometric mean RSV titre of the group, with the viral titre number in parentheses. The horizontal hatched line, across the graph, represents the RSV titre at the limit of assay detection.
In this study, we demonstrate that the known Smo-receptor antagonists cyclopamine and jervine are potent inhibitors of hRSV infection. Cyclopamine targets the viral replication complex via the processivity factor M2-1; this is the first validation of M2-1 as a target for therapeutic intervention against hRSV. It identifies a novel therapeutic potential for cyclopamine and previously described analogues.

The Smo antagonist cyclopamine potently inhibited post-entry events of hRSV replication in vitro, but the structural analogue tomatidine, which does not bind to Smo, had no effect on infection. Several Veratrum alkaloids were evaluated in vitro to assess the structure-activity relationship of cyclopamine. Only jervine, another Smo antagonist [36], blocked viral replication. Cyclopamine is also called 11-Deoxojervine, for it only lacks a keto group in C-11 position as compared to jervine. The keto-group in C-11 leads to an approximately 30-fold shift in IC_{50}. Further structure activity relationship assessments will entail the analysis of cyclopamine analogues.

We hypothesised that Shhp inhibition could be the reason for cyclopamine’s antiviral effect. We tested the smoothened inhibitors GDC-0449, approved under the name Vismodegib, and LY2940680, currently in clinical trials, as well as the smoothened activator purmorphamine. These three molecules were shown to bind to the same region as cyclopamine on Smo. GDC-0449 and LY2940680 are Smo antagonists and compete with cyclopamine [23,33,37], while the SMO-agonist purmorphamine counteracts the inhibitory effect of cyclopamine [35,38]. GDC-0449, LY2940680 and purmorphamine had no effect on infection in vitro, neither individually nor in combination with cyclopamine. A monoclonal antibody directed against the extracellular...
Sonic hedgehog ligand was previously shown to slightly reduce HCV replication [39], but it had no effect on hRSV replication (data not shown). Altogether these data indicate an off-target effect of cyclopamine which does not involve the Smo receptor.

In order to identify the mechanism of action of cyclopamine, we generated compound-resistant mutants by resistance selection. A single point mutation in the hRSV genome at the R151K position of the M2-1 protein was able to confer resistance to cyclopamine. Our data are consistent with previous studies which demonstrated that this residue of M2-1 is critical for replication and showed that a R151D mutation results in 70% reduction of M2-1-dependent transcription [18,40]. To confirm that the replication complex was indeed the target, we further utilised a minigenome assays to confirm the inhibitory activity of cyclopamine on hRSV replication complex. Cyclopamine was very potent against hRSV RdRp activity in the minigenome assay, with an IC₅₀ of 380 nM, while the M2-1 R151K mutant was significantly less sensitive. Inhibition reached a plateau of 26% at 1 µM cyclopamine. This result demonstrated that the R151K mutation is responsible for cyclopamine resistance. R151 is conserved in M2-1 sequences of animal and human Pneumo- and Metapneumoviruses (Supplementary Figure 1). This indicates that pre-existing resistant R151K viruses are unlikely to circulate in the population. These results are also consistent with the observation that cyclopamine had no effect on hPIV-3, which does not encode an M2-1 protein.

CPM reduced M2-1 expression levels in minigenome-transfected cells expressing M2-1WT, which was not the case when M2-1R151K was expressed. The overall levels of M2-1R151K, however, were lower than the ones of M2-1WT. This could be due to a poorer stability of the mutant protein, or a lower overall expression resulting from the mutation. However, M2-1 could still be detected in inclusion bodies in the presence of CPM. Both phosphorylated and non-
phosphorylated forms of M2-1WT are significantly reduced in a dose-dependent manner compared to M2-1R151K. Phosphorylation of M2-1 is critical for its function [40–42]. M2-1 is phosphorylated at S58 and S61 during the hRSV life cycle [42], and their substitution by alanine reduces M2-1 anti-termination activity [40]. Furthermore, mimicking phosphorylation at these residues with phosphomimetic S58D and S61D significantly reduces RNA transcription. Thus, it is likely that the anti-termination function of M2-1 depends on its ability to dynamically switch phosphorylation states, as allowed by reversible phosphorylation. One hypothesis for the requirement for dynamic phosphorylation is that transcription anti-termination is a multistep process, at least one of which requires the phosphorylated state and, conversely, at least one other step requires M2-1 in the unphosphorylated state. These steps could include the binding of ligands, such as viral RNA gene-end sequences, which consequently allows the recycling of various forms of M2-1 into or away from the transcribing polymerase [40]. S58 and S61 are located in a flexible, disordered loop which is close to R151. Thus, the present results suggest that there is a dialog between the RNA- and P-binding domains of M2-1 in which R151 is located and the phosphorylatable loop containing S58 and S61. Phosphorylated or unphosphorylated states could also induce a change in local charges and influence interactions between M2-1 and RNA or P. However, how CPM could interfere with these mechanisms remains to be clarified. It is necessary to understand the mechanisms by which M2-1 is phosphorylated and dephosphorylated and by which partner, which still remain unknown. Furthermore, in a recent study, it was shown that the tetrameric M2-1 protein of human metapneumovirus (HMPV), which is very similar to RSV M2-1, can adopt different conformations [43]. This dynamic equilibrium between the open and closed states of M2-1 is likely to be involved in the RNA-binding process of the protein.
R151 is located in the region responsible for binding to RNA preferentially, although it can also bind P. However, we did not observe an effect of CPM on either P- or RNA-binding to M2-1, even in the presence of large excess of CPM, suggesting that CPM may not bind directly to the protein. This finding was confirmed by solution-based NMR, which revealed no perturbation to the spectrum of the M2-1 core domain (residues 59-177) after addition of excess amounts of cyclopamine (data not shown). It has been determined previously that M2-1 binds specific RNA sequences with high affinity, these sequences corresponding to RSV gene end (GE) or polyA [18,40]. Since we used tRNA in our in vitro experiments, we cannot exclude than CPM could interfere more specifically with GE or poly-A RNAs, or that another more complex mechanism is responsible for resistance of R151K mutant to CPM. hRSV replication is a highly complex process involving 4 viral proteins, viral RNA and cellular co-factors. To precisely determine the molecular mechanism of action of CPM, and to investigate its potential direct binding to M2-1 would require the development of novel biochemical assays that reconstitute the hRSV replication complex in vitro.

Cyclopamine is able to reduce lung virus titres by 1.5 logs in the BALB/c model of hRSV infection. Several other small molecules have been developed for hRSV infection and are at various stages of development, as animal or human proofs of concept. The most advanced compounds are inhibitors of the critical viral fusion mediated by the virus F protein. The compound GS-5806, a pyrazolo-pyrimidine compound, has been shown to have efficacy in phase 2a clinical trials and reduced both viral load and clinical symptoms of disease [44]. Wyde et al described another fusion inhibitor in a cotton rat model of infection, the bis-tetrazole-benzhydrophenol compound VP14637 which reduced RSV lung titres by over 2 logs and reduced histopathological changes due to RSV infection [14]. Although chemically unrelated,
the azabenzimidazole compound BMS-433771 also targets the fusion protein. When
administered orally in the BALB/c mouse model, BMS-433771 was able to reduce lung titres of
hRSV by 1.5 logs, reaching the detection limit of the assay [45]. Results of data obtained in the
mouse model were predictive for efficacy in the cotton rat model by the same group, where the
compound efficacy was proven despite different pharmacokinetic and pharmacodynamics
properties between the mouse and the cotton rat models [31].

Our data open a promising new avenue in the search for a safe and efficacious therapy of hRSV
infection. Further development of steroidal alkaloids as hRSV therapeutics will require the
development of novel synthetic chemistry, or the identification of naturally occurring molecules
that inhibit the M2-1-dependent hRSV replication but do no longer bind to the Smo receptor.

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References


Supplementary Figure 1. Sequence alignment of M2-1 of human respiratory syncytial virus (HRSV), bovine respiratory syncytial virus (BRSV), ovine respiratory syncytial virus (ORSV), pneumonia virus of mice (PVM), canine pneumovirus (CPV), human metapneumovirus (HMPV), and avian metapneumovirus (AMPV). Alignments were generated using the Clustal Omega program and displayed with the program ESPript [46].

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Chapter 4

Drug repositioning for the treatment of enterovirus 71 infection

4.1 Preface

Enterovirus 71 is an important emerging pathogen responsible, together with Coxsackie virus A16, for hand, foot and mouth disease (HFMD) in children. While usually benign, the disease sometimes develops into severe neurological disorders and can cause death. Since the discovery of HFMD in 1957, EV71 has been the cause for several outbreaks predominantly in Asian countries such as China, Vietnam and Taiwan, with several million reported cases. Despite this burden, no specific treatment is available for EV71 infection or HFMD, and patients only have access to palliative medicines. Given the potential severity of symptoms associated with EV71 infection, targeted-therapies are desperately needed.

In an attempt to respond to this urgency, the study reported in this Chapter was based on the idea that repurposing existing drugs for the treatment of EV71-associated HFMD could be the most efficient way to advance compounds to clinical trials.

A phenotype-based screening of an approved drug library on EV71 infection in vitro was therefore carried out in the attempt to identify inhibitors that could be repurposed for the treatment of HFMD. From this screening, a hit was identified and was evaluated for its capacity to inhibit replication in vitro of several clinical isolates of the C4 genotype,
from China and Cambodia. It was also evaluated for effect on CV-A16 and poliovirus infection, and showed antiviral efficacy for the former but not the latter. The drug-hit was tested on infection in vitro at different stages of infection, and revealed a higher potency when present during virus adsorption to the host cell. As the hit is a polysulfonated drug, several sulfated and sulfonated structural analogues were assayed in vitro and proved to have antiviral properties against EV71. Since the virus has the ability to bind heparan sulfate, and the drug is polysulfonated, STD-NMR experiments using whole virus particles in presence of drug were carried out to verify if the virus capsid was the molecular target, in an attempt to validate the compound as an entry inhibitor of EV71 infection. Finally, the PK/PD of the drug was evaluated in the cynomolgus monkey model, as well as EV71 antiviral efficacy in the suckling mouse and rhesus monkey models of infection.

Together, these data present the potential of the hit to be used as a prophylaxis and treatment for EV71-associated HFMD. It is an approved paediatric drug that has good potential for further clinical investigations.

References

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Statement of Contribution to Co-Authored Published Paper

This Section includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:


My contribution to the paper involved:

1. The conception and design of STD-NMR experiments.
2. The acquisition and interpretation of data related to 1.
3. The preparation of Figure 4.
4. The review of the manuscript.

Permission to use the paper in this Thesis has been obtained from Nature Publishing Group.

The Supplementary Material relevant to this paper can be found in Appendix 6.3, p. 243.

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The approved pediatric drug suramin identified as a clinical candidate for the treatment of EV71 infection—suramin inhibits EV71 infection in vitro and in vivo

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Enterovirus 71 (EV71) causes severe central nervous system infections, leading to cardiopulmonary complications and death in young children. There is an urgent unmet medical need for new pharmaceutical agents to control EV71 infections. Using a multidisciplinary approach, we found that the approved pediatric antiparasitic drug suramin blocked EV71 infectivity by a novel mechanism of action that involves binding of the naphthalenetrusonic acid group of suramin to the viral capsid. Moreover, we demonstrate that when suramin is used in vivo at doses equivalent to or lower than the highest dose already used in humans, it significantly decreased mortality in mice challenged with a lethal dose of EV71 and peak viral load in adult rhesus monkeys. Thus, suramin inhibits EV71 infection by neutralizing virus particles prior to cell attachment. Consequently, these findings identify suramin as a clinical candidate for further development as a therapeutic or prophylactic treatment for severe EV71 infection.

Keywords: anti-viral; drug discovery; enterovirus 71; hand, foot and mouth disease; suramin

INTRODUCTION

Hand, foot and mouth disease (HFMD), a contagious infectious disease mostly affecting children under the age of five years, is common in Asia¹–³ and has been particularly prevalent since 2008.⁴ The disease is endemic in other regions but severe forms are rarely observed.⁵ In China alone, over 7.6 million children have been diagnosed with HFMD and more than 2400 of these children have died, since China alone, over 7.6 million children have been diagnosed with HFMD and more than 2400 of these children have died, since 2008.⁶,⁷ In most cases, symptoms are mild, such as fever, malaise, rashes on the hand palms, soles of feet, buttocks and herpangina. However, severe disease, including central nervous system infection, brain stem encephalitis, neurogenic pulmonary edema and cardiopulmonary complications, may occur and is frequently fatal.² Children become susceptible to severe EV71 infections after the loss of maternal antibodies and one to two-year-old children are most at risk.⁸

EV71 is a single-stranded positive-sense RNA virus from the Picornaviridae family, genus Enterovirus (along with Poliovirus and coxsackievirus species A).⁹ It has a non-enveloped capsid consisting of 60 identical subunits, each containing one copy of each of four viral structural proteins (VP1, 2, 3 and 4), and packages a 7.5 kb genome. EV71 can be classified into genogroups A, B and C, recently identified D, E and F⁹ on the basis of its VP1 gene sequence. Group C is prevalent in East Asia and the C4 genotype currently predominates in mainland China, Vietnam, Cambodia and prevalent in Taiwan and Thailand.¹⁰

During infection, EV71 binds to host cells via viral receptors, such as human scavenger receptor class B, member 1¹¹ and P-selectin glycoprotein ligand-1.¹² Binding to scavenger receptor class B, member 2 triggers the uncoating process,¹³ a series of structural changes occurring in the viral capsid leading to the release of the viral genome into the host cell. Like many other viruses, EV71 also uses cell surface heparan sulfate glycosaminoglycan as attachment receptor to initiate target cell entry.¹⁴ Also recently, Du et al.¹⁵ demonstrated that cell surface vimentin serves as an attachment site that mediated the initial binding and subsequently increased the infectivity of EV71.

There is currently no specific anti-EV71 drug, and guidelines for the treatment of HFMD are therefore limited to supportive care, antipyretic drugs, intravenous non-immune immunoglobulin and possibly, glucocorticoids.¹⁶ Type 1 interferons and inhibitors of 3C protease, 3D polymerase and entry inhibitors are candidate drugs for the treatment of EV71 infections. However, no proof-of-concept study has yet been established for these treatments in non-human primate models or clinical trials.

A series of structurally related antiviral compounds known as the Winthrop compounds inhibit picornavirus attachment to host cells...
and virus uncoating, by binding to a hydrophobic pocket of the capsid. The Winthrop compound pleconaril, attenuates severe symptoms in EV71-infected mice, although differences in potency between viral isolates were described. Pleconaril and Winthrop compounds served as scaffolds for the design of pyridyl imidazolidinoliones. Two of these compounds, BPR0Z-194 and DBPR-103, have potent antiviral activity, preventing the attachment or uncoating of several enteroviruses, including EV71. A proteinase inhibitor, has potent broad-spectrum activity against human rhinovirus and human enterovirus, including EV71, both in vitro and in vivo. Ribavirin, which can be imported by viral RNA-dependent RNA polymerase, is sometimes used to treat HFMD. Glycosaminoglycans such as heparin, heparan sulfate and their mimetics have been shown to strongly inhibit EV71 attachment to cells suggesting that interfering with EV71 binding to glycosaminoglycans could be used as a target for the development of an antiviral.

We investigated whether any United States Food and Drug Administration-approved drugs were of potential value for treating EV71 infection. There are several advantages to focusing on approved drugs: (i) experience in clinical use or data from clinical trials, for pharmacokinetics and toxicity in particular, can significantly decrease development time; and (ii) the physiological roles of the targets of most approved drugs are known, facilitating mechanism-of-action studies and providing valuable information about potential drug–drug interactions.

We identified suramin as a clinical candidate molecule directly binding the EV71 capsid, blocking attachment and entry and decreasing viral replication in susceptible animals. Suramin has been in clinical use for decades as a prophylactic and therapeutic agent in children. Attempts have recently been made to develop the use of suramin in a cancer setting and as an antiviral agent against human immunodeficiency virus and hepatitis B virus. We identified suramin as an inhibitor of EV71 entry and provide the first demonstration of the efficacy of a small molecule in a non-human primate model of EV71 infection.

MATERIALS AND METHODS

Cell lines and viruses

RD (human rhadinoblastoma) cells were purchased from the American Type Culture Collection (ATCC NO CCL-136). The EV71 isolate Fuyang573 (subgenotype C4a, GenBank accession number: HM064456, isolated from a 2008 epidemic sample in Anhui province) was used throughout this study, unless otherwise stated. EV71 isolates SH12-036 (GenBank accession NO KF543271) and SH12-276 (GenBank accession NO KF543271) were provided by the Virology Unit of the Institut Pasteur in Cambodia. Coxackie virus A16 (strain shzh05-1, GenBank accession NO EU262658) and poliovirus-1 (Sabin, type I oral vaccine) were also used to evaluate antiviral potency. We titrated virus stocks on RD cells, by both microtitration tissue culture infective dose 50% (TCID50), according to the Kärber formula and plaque assays in 0.7% carboxymethylcellulose.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for EV71 viral load quantification

We extracted RNA with the TIANamp RNA Extraction Kit for Virus Detection (cat. NO DP315-R; Tiangen Biotech Beijing Co., Ltd, Beijing, China), or the TIANamp N96 Virus RNA Kit (cat. NO DP434; Tiangen Biotech Beijing Co., Ltd) in semihigh-throughput operations. We assessed viral genome load with the Quant One Step qRT-PCR (Probe) Kit (cat. NO FP504; Tiangen Biotech Beijing Co., Ltd) on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The VP1 gene was detected with forward primer: 5'-GGT TAC CAT CTA CAT GCG CCT TTA G-3' and reverse primer: 5'- TGG AGA TAC TGT GGA ACA AC-3' and probe: 5'-HEX-TCT TGC GTG CAC ACC CAG TAMRA-3'. The PCR standard curve was obtained by serial dilution of the defined-titer (TCID50/mL) virus stock, and the sample cycle threshold (Ct) number was converted into viral load with this standard curve (Supplementary Figure S1), and viral load is expressed as EV71 genome equivalent.

Cell viability assay

We evaluated cell viability with the CellTiter-Glo Luminescent Cell Viability Assay Kit (cat. NO G7571; Promega, Fitchburg, WI, USA).

Drug library and compounds

We screened the United States Drug Collection (1040 compounds) and the International Drug Collection (240 compounds) (MicroSource Discovery Systems Inc., Gaylordsville, CT, USA), searching for compounds active against EV71.

Suramin sodium salt (cat. NO S2671), PPADS (pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate, cat. NO P178), vinylsulfonic acid sodium salt (cat. NO 278416) and heparin sodium salt (cat. NO H3392) were purchased from Sigma-Aldrich (St. Louis, MO 63103, USA). DDIDS (4,4′-dissulfoanostilbene-2,2′-disulfonic acid disodium salt, cat. NO sc-20391) was obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA 95060, USA). iso-PPADS tetrasodium salt (pyridoxal phosphate-6-azophenyl-2,5′-disulfonic acid tetrasodium salt, cat. NO 0683), NF 023 (8,8′-[carboxybis(imino-3,1-phenylene)carbonylimino][bis-1,3,5-naphthalenetrisulfonic acid, hexa- sodium salt, cat. NO 1240], NF 157 (8,8′-[carboxybis(imino-3,1-phenylene)carbonylimino][bis-1,3,5-naphthalenetrisulfonic acid hexa-sodium salt, cat. NO 2450) and NF 449 (4,4′,4″-[carboxybis(imino-5,1,3-benzentriyl-bis(carboxyli- mino)])[tetrazis-1,3-benzenedisulfonic acid, octasodium salt, cat. NO 1391) were obtained from Tocris Bioscience (Bristol, UK). Sucralfate sodium was obtained from MicroSource Discovery Systems Inc. Suramin used in the monkey study was provided free-of-charge by Bayer Healthcare (Elberfeld, Germany).

Drug screening

We screened the drug library in 96-well plates, by inoculating 5×10^5 RD cells per well with 10 μM drugs and incubating at 37°C for 1 h. We then infected cells, at a multiplicity of infection of 0.1, in the presence of 10 μM drugs, at 37°C for 1 h. The cells were then incubated, in the presence of 10 μM drug, at 37°C for 46–48 h, under an atmosphere containing 5% CO2. We collected the culture supernatant, extracted the viral RNA and determined viral load by qRT-PCR.

Antiviral potency assay

The cells and the virus were incubated separately with the compound for 1 h at 37°C. The cells were then infected in the presence of the compound for 1 h and incubated with the compound for 46–48 h. We then evaluated viral load by qRT-PCR. Antiviral potency was also evaluated by microtitration (results expressed as TCID50/mL) with a series of concentrations of the compound assessed. Additionally, plaque assays were carried out, in which we incubated 90% confluent RD cells and virus separately with the compound and then infected cells with 50 plaque forming units EV71 in the presence of the compound.
In vivo anti-EV71 efficacy
The anti-EV71 efficacy of suramin in vivo was assessed in 10-day-old Institute of Cancer Research mice and adult rhesus monkeys, as previously described. We injected 1 × 10^9 TCID_{50} (lethal dose) of the mouse-adapted EV71 strain MP10 (GenBank accession NO HQ712020, genotype C4) intraperitoneally into mice. We then injected 20 or 50 mg/kg suramin dissolved in saline, or saline alone as a placebo, intraperitoneally into the mice, twice daily for seven days. For monkey studies, we intravenously injected 1 × 10^9 PFU cell culture infective dose 50% (CCID_{50}) EV71 FY-23 (GenBank accession NO EU812515, genotype C4) into the monkey. We then injected 50 mg/kg suramin in saline, or saline alone as a placebo, into the monkeys intravenously on the day before virus challenge and on days 1, 3, and 5 post-challenge. We then assessed serum viral load by qRT-PCR, assessed the neutralizing antibody titer on RD cell as described before, in neutralizing assay, serum was diluted for eight times.

Saturation transfer difference nuclear magnetic resonance spectroscopy (STD NMR)
We prepared viral particles for the STD NMR assay by inactivating the virus stock by incubation with the virus stock by incubation with 1:2000 (v/v) β-propiolactone (100168; TCJ, Shangh, China) overnight at 4°C. We then concentrated the viral particles by centrifugation on a 20% sucrose cushion, and the pellet was resuspended in phosphate-buffered saline, ultracentrifuged on 10% sucrose, sedimented, and resuspended two times. The purified virus was as described previously for EV71.

Cytochrome P450 (CYP) inhibition assay
CYP inhibition was determined with a marker substrate cocktail. For each reaction, enzyme activities in the presence and absence of the test compound (10, 30, and 100 μM) were measured in duplicate. Known inhibitors for each isoenzyme (CYP1A2, CYP2C9, CYP2C19, CYP3A4, CYP3A5) were used as positive controls.

Incubation mixture containing human microsomes, substrate cocktail and standard inhibitor or test compound was pre-incubated at 37°C for 5 min. The reaction was initiated by adding nicotinamide adenine dinucleotide phosphate. The mixture was incubated at 37°C for 10 min, and ice-cold acetonitrile was added to terminate the reaction. We assessed metabolite generation from the substrate reactions by liquid chromatography-tandem mass spectrometry and peak area ratios for analyte/internal standard. The extent of inhibition was expressed as a % of control activity.

Cynomolgus monkey plasma pharmacokinetics
We studied the plasma pharmacokinetics of suramin in male cynomolgus monkeys. Three monkeys were given 4.37 mg/kg body weight suramin by intravenous bolus administration, with serial blood sample collection for up to seven days. Plasma samples were obtained by centrifugation (3000 g for 10 min at 2-8°C). A liquid chromatography-tandem mass spectroscopy method was developed for the quantification of suramin in monkey plasma. Changes in plasma concentration over time were analyzed with a non-compartmental model in WinNonlin software (version 5.2.1; Pharsight, Mountain View, CA, USA), with calculation of the following pharmacokinetic parameters: AUC_{last}, AUC_{inf} (AUC: area under the concentration time curve; AUC_{last} AUC up to the last measurable concentration; AUC_{inf} AUC curve to infinite time), half-life (T_{1/2}), maximum concentration observed (C_{max}), clearance (CL), volume of distribution calculated either by the steady-state method (V_{dss}).

RESULTS
Approved drug library screening
One thousand two hundred and eighty drugs from the United States and International Drug Collection were screened, at 10 μM, using EV71 genome equivalent reduction in the supernatant of infected RD cells by >1 log_{10} and cytotoxicity less than <25% as readout. Suramin was selected for further analysis based on its inhibition profile and its approval status as a pediatric drug. Suramin inhibited several C4-genotype EV71 isolates (Figure 1A) with a concentration causing 90% inhibition (IC{90}) of 0.9, 3.92, 22.19 and 25.84 μM for the Fuyang573 (Anhui 2008), SH12-036, SH12-276 (Shanghai 2012) and SEP-4 (Cambodia 2012) isolates, respectively. These results were confirmed in an EV71 plaque reduction assay, in which the IC{90} of suramin was 0.49, 6.08 and 7.80 μM for Fuyang573, SH12-036 and SH12-276, respectively (Figure 1B). Suramin was not cytotoxic at concentrations as high as 1 mM (Figure 1A) and had a selectivity index greater than 12 500. In TCID_{50} reduction assays, coxsackievirus A16 replication was reduced by 10^{6} TCID_{50}/mL by 50 μM suramin, whereas poliovirus-1 (Sabin) was not inhibited (Supplementary Figure S2).

Suramin inhibits EV71 entry
We investigated the step in the viral infectious cycle targeted by suramin, by time-of-addition assays in which cells and virus were pre-incubated or not with 10 μM suramin, and 10 μM suramin was present or not in viral-cell adsorption and post adsorption stage of EV71 infection. Single round viral replication is get by infecting RD cell at multiplicity of infection of 5, collecting at 16 h post infection, and testing culture supernatant and intracellular RNA at 16 h post infection. Suramin decreased viral replication by >1 log_{10}, when added at the virus-cell adsorption stage, but had no effect if added after adsorption (Figure 2A). Furthermore, when incubated with cells and virus at 4°C, to prevent virus internalization, suramin reduced virus binding to the cell with an IC_{90} of 6.17 μM (Figure 2B).

Sulfonated and sulfated compounds inhibit EV71 infection
Structural analogs of suramin also inhibited EV71 replication (Figure 3A), with the following IC_{90}: NF 449, 0.9 μM; NF 157, 2.5 μM; iso-PPADS, 6.4 μM; PPADS, 7.0 μM; NF 023, 8.9 μM. Sulfated and sulfonated compounds that were not structural analogs of suramin were also shown to be active: the monosulfonated compound vinylsulfonic acid sodium and the disulfonated compound DIDS inhibited EV71 infection with an IC_{90} of 4.5 μM and
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10.3 μM, respectively, and the polysulfated molecules, heparin and sucralfate sodium, inhibited EV71 replication with IC90 values of 24.3 μg/mL and 3.3 μM, respectively.

STD NMR is a powerful tool for assessing small-molecule binding to viruses. We observed strong STD NMR signals for all protons on the suramin framework (Figure 4A), whereas the proton signals of H7 and H8 protons, displayed the strongest signal intensities, indicating close proximity of these protons and consequently that part of the molecule to the EV71 capsid (Figure 4B).

Our results indicate that suramin binds to the EV71 particle via the naphthalenetrisulfonic acid group, preventing viral attachment and entry.

Pharmacokinetics of suramin

CYP1A2 was the only CYP enzyme tested to display slight inhibition by suramin, with an IC50 of 10 μM (Supplementary Table S1), suggesting a low risk of drug–drug interaction. Suramin did not inhibit the human Ether-a-go-go-related gene channel (Supplementary Table S2), suggesting a low likelihood of cardiotoxicity.

Figure 1

Suramin was identified as an EV71 inhibitor. (A) Suramin inhibits the replication of EV71 isolates Fuyang573, SH12-036, SH12-276 and SEP-4, without cytotoxicity. Viral load was measured by quantitative RT-PCR, and expressed as the EV71 genome equivalent to TCID50/mL. Data represents the mean ± s.e.m. of results of duplicated experiment. (B) Suramin reduces the progeny virus yield. Data represents the means ± s.e.m. of results of two independent experiments which are duplicated.

Figure 2

Suramin blocks EV71 virus-cell attachment. (A) Time of addition assay. 10 μM suramin was added at different stage in viral infection as shown in figure. The viral load in supernatant represents the mean ± s.e.m. of results of experiment with three replicates, and the intracellular viral load represents the result of a single test. (B) Inhibition of viral attachment by suramin (cell-virus adsorption at 4C). Data represents the means ± s.e.m. of results of experiment with three replications.

The approved dose of suramin is 1 g for adults and 10–15 mg/kg for children (http://home.intekom.com/pharm/bayer/suramin.html). We used 15 mg/kg as the highest human reference dose. Following a single intravenous administration of 4.37 mg/kg suramin, corresponding to one-eleventh the highest human dose allometrically scaled to the monkey (46.5 mg/kg), in male cynomolgus monkeys, suramin was rapidly detected in the plasma and cleared slowly with an average CL of 0.0317 mL/min/kg (Supplementary Figure S3 and Supplementary Table S3). Suramin plasma level reached 10.9 μM at 24 h (Supplementary Figure S3 and Supplementary Table S4), which is >10 times superior to the in vitro IC50 (0.93 μM to Fuyang573 isolate) (Figure 1). Plasma drug level is 2.9 times to the IC50 at seven days (168 h) after a single-dose administration.

Suramin efficacy

We assessed the suramin efficacy in 10-day-old Institute of Cancer Research mice infected with lethal doses of the mouse-adapted EV71 strain MP10. Treatment with 50 mg/kg resulted in survival rates of 30% while vehicle-treated mice developed paralysis at 3 dpi and died within 10 days of infection (Figure 5A).

Rhesus and cynomolgus monkeys can be successfully infected with EV71 and represent the most predictive animal models for EV71. Suramin monkeys were treated four times with 50 mg/kg suramin, the drug level is 2.9 times to the IC90 at seven days (168 h) after a single-dose administration.
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Figure 3 Sulfonated and sulfated compounds inhibit the replication of the EV71 Fuyang573 isolate. (A) Sulfated suramin analogs inhibit EV71 infection. Data represents the means ± s.e.m. of results of experiment with three replications. (B) Sulfonated and sulfated compounds not analogous to suramin inhibit EV71 infection. Data represents the means ± s.e.m. of results of experiment with three replications.

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DISCUSSION

Drug development for acute pediatric infectious diseases is challenging due to long development times and high costs. We reasoned that the repurposing of approved drugs, particularly those for pediatric use, might be a useful approach. We found that suramin, previously approved for the treatment and prophylaxis of African sleeping sickness and onchocerciasis, inhibited EV71 replication in vivo at doses at or below the highest human dose.

HFMD epidemics occur annually in several Asian countries. Physicians are faced with large numbers of patients with mild symptoms (rash, fever) and a lack of markers of progression to severe disease, which typically occurs one to two days after symptom onset. There are two major therapeutic needs: (i) treatment of children diagnosed with EV71, to prevent progression to severe forms and death; and (ii) prophylactic treatment of children in contact with EV71-infected children, to prevent viral transmission.

Suramin was the only drug, approved for prophylactic and therapeutic uses in children, identified in our screening campaign. Significant toxicity has been observed in patients with Trypanosoma infection, due to inflammatory reactions caused by suramin-mediated...
killing of parasite.\textsuperscript{39} However, data from patients without parasitic infections\textsuperscript{40–42} suggest that suramin is generally safe, provided that plasma drug concentrations do not exceed 200 $\mu$M.\textsuperscript{40} Furthermore, suramin had no significant effect on Ether-a`-go-go-related gene channel activity and little potential for drug–drug interactions.

Suramin has a long half-life\textsuperscript{25,43} and $10$ times the IC$_{90}$ for EV71 can be reached in monkeys after a single injection at one-eleventh the highest human dose allometrically scaled to monkeys according to United States Food and Drug Administration guidelines. This profile makes a single-dose strategy possible, with sufficiently high drug concentrations being reached over a few days following a single injection and ensuring antiviral efficacy throughout the period of peak viremia.\textsuperscript{30,35,44} Most antiviral drugs target viral enzymes involved in replication, but viral entry has been successfully used as a target for antiviral drug development for human immunodeficiency virus.\textsuperscript{45–47} Pleconaril, which binds the capsid of human rhinovirus, a picornavirus, prevents virus entry.\textsuperscript{48} It was tested in phase III trials for common cold treatment, but did not obtain regulatory approval. Pleconaril is also active against EV71 but its potency varies considerably between viral isolates.

The mode of action of suramin involves the sulfonate groups of the naphthalene moiety. Our results are consistent with those of Tan et al\textsuperscript{14,22} who simultaneously described sulfate-mediated inhibition of EV71 entry by demonstrating the binding of EV71 to cell surface heparan sulfate glycosaminoglycan and the blocking of this binding by

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{STD NMR assay of suramin-EV71 particle binding. (A) STD NMR of suramin with the EV71 particle. $^1$H (proton) NMR spectrum of suramin, shown in black, and STD NMR spectrum of suramin in complex with EV71 particles, shown in red. (B) Left: structure of suramin, labeled with proton positions and relative intensity percentages. Right: $^1$H NMR spectrum of suramin, with proton numbers shown.}
\end{figure}
suramin. NF449, a suramin analog, has also been shown to inhibit EV71. A large number of sulfated and sulfonated molecules inhibit EV71 (Figure 3), including several antagonists of P2X receptors (Figure 3A), suggesting a possible role for P2X receptors in cell entry. However, no non-sulfonated/sulfated P2X inhibitors displayed activity (Figure 3B) and the siRNA knockdown of P2X receptors did not decrease viral replication or affect the ability of suramin to block EV71 replication (data not shown).

Time-of-addition and virus binding assays showed that suramin prevented EV71 from binding to the target cell in vitro (Figure 2). STD NMR spectroscopy is a powerful tool for identifying the pharmacophores of small molecules binding to virus particles. Our study of suramin in complex with EV71 particles by this technique clearly demonstrated that the protons adjacent to the viral capsid are positioned close to the sulfonic acid groups, identifying the naphthalene trisulfonic acid group as the pharmacophore by which suramin inhibits virus attachment and replication (Figure 4).

Mechanism-of-action studies suggested that suramin inhibited virus entry through a mechanism similar to the antibody-mediated neutralization of virus particles.

**Figure 5** Anti-EV71 efficacy in vivo. (A) Infect 10-day-old ICR mice with 1×10^7 TCID_{50} (lethal dose) of the mouse-adapted EV71 strain MP10. Then inject 20 or 50 mg/kg body weight suramin i.p. injection, twice daily for 7 days. There are 10 mice in each group. (B) Suramin at a dose of 50 mg/kg body weight decreases EV71 viremia in adult rhesus monkeys. Challenge adult monkeys with 1×10^7 TCID_{50} EV71 FY-23 strain, and inject 50 mg/kg suramin i.v., on the day before virus challenge and on days 1, 3 and 5 post challenge. There are five monkeys in each group.

We evaluated suramin efficacy in two validated animal models. In mice, suramin decreased mortality by 30% (Figure 5A). In the monkey model, previously shown to be of predictive value in vaccine development, the highest human dose of suramin, allometrically scaled to the monkey decreased peak viremia (Figure 5B). This provides the first proof-of-concept that a small-molecule inhibitor can have a strong antiviral effect against EV71 in non-human primates.

Suramin displays high levels of serum protein binding, generally considered predictive of poor therapeutic efficacy for small molecules. However, our data suggest that the protein-binding features of suramin may be a key element in its anti-EV71 activity and that circulating EV71 may be neutralized by suramin in the blood.

The primary objective of the treatment of EV71 infection is preventing severe and fatal outcome of disease. Our findings suggest that suramin, an approved pediatric drug, may be useful for therapeutic and prophylactic applications in young children infected with or exposed to EV71. Overall, this study indicates that the identification of new indications for approved drugs is an attractive approach for developing new treatments for acute viral infections in situations of major unmet need. Moreover, we believe that our study supports the notion that suramin presents an exciting opportunity as a possible drug candidate to treat and prevent HFMD and severe EV71 infections. This opportunity should be investigated further, by evaluating safety and efficacy in clinical studies.

**ACKNOWLEDGMENTS**

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Chapter 4. Inhibitor of EV71 infection

Suramin inhibits EV71 infection in vitro & in vivo
PJ Ren et al


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Chapter 5

Conclusion and Future Directions

The research project presented in this Thesis was composed of four studies, that were designed around the discovery of novel inhibitors of paediatric viral pathogens using multidisciplinary approaches. The targets were principally associated with the paramyxoviruses hPIV-3 and hRSV, and extended to the picornavirus EV71.

Neu5Ac2en-based compounds, potent competitive inhibitors of hPIV-3 HN

The first study reported the structure-based discovery of novel inhibitors of hPIV-3 HN in vitro by rational, computer-aided drug design (Section 2.2, p. 79). Based on the crystal structure of hPIV-3 HN in complex with zanamivir, several analogues of Neu5Ac2en with modifications at the C-4 and C-5 positions were designed and synthesised. They were shown to better occupy the catalytic site of the protein, by filling the cavity around the 216-loop while locking it open via engagement of a phenyltriazole moiety at the C-4 position that engages the protein and enhances the inhibitors avidity. As a consequence, these compounds demonstrated low μM potency against the haemagglutinin and neuraminidase functions of HN, and against hPIV-3 infection in vitro.

The most potent molecule that was designed and synthesised contained an isobutryramido group at the C-5 position, and a phenyltriazole group at the C-4 position. The compound is to date the most potent inhibitor of hPIV-3 HN, and it demonstrates the power of rational inhibitor design to produce potent antivirals.
Although compound 10 achieved low-micromolar potency \textit{in vitro}, it may not be sufficient for it to be considered as a drug-candidate as a nanomolar inhibitor may be more suitable. Nevertheless, this compound presents as a lead-like inhibitor for further development of competitive hPIV-3 sialidase inhibitors. To further improve on the present discovery, a crystal structure of hPIV-3 HN in complex with compound 10 would be of tremendous value. Apart from validating the computational simulations that were carried out in this project, such structures would allow the precise design of refined inhibitors, taking into consideration key interactions between the protein and the inhibitor as well as the open-state of the 216-loop.

Compound 10 was tested \textit{in vitro}, both in monkey and human-derived immortalised cell-lines, but not in \textit{in vivo}. Given the difficulty to work with small animal models of hPIV-3 infection like the mouse or the cotton rat, lead compounds such as those reported in this study could be tested in an \textit{ex-vivo} differentiated-pseudostratified human airway epithelial cell model of infection to preliminarily validate their \textit{in vivo} efficacy and toxicity. These cells, ciliated and producing mucus, are grown at the air-liquid interface and mimic the respiratory tract environment. This method has proven successful in the past, and in the case of hPIV-3 was shown to replicate infection of the cotton rat model\textsuperscript{2–4}.

**Suramin, a non-competitive inhibitor of hPIV-3 HN**

The second study reported the discovery of the approved drug suramin as an inhibitor of hPIV-3 infection \textit{in vitro} (Section 2.3, p. 91). The drug was selected by target-based, enzymatic screening of an approved drug library on hPIV-3 HN neuraminidase activity, and was shown to also inhibit the haemagglutinin activity of the protein. Therefore, suramin was confirmed as both an entry and release inhibitor of infection \textit{in vitro}. The mechanism of action of suramin was characterized by enzyme kinetics, competition STD-NMR spectroscopy and \textit{in silico} docking simulations, and led to the conclusion that the drug is a non-competitive inhibitor of the hPIV-3 HN. Following this discovery, it was demonstrated that suramin has the ability to work in synergy with competitive inhibitors of the hPIV-3 HN to block infection \textit{in vitro}.
Suramin is a drug currently approved for the treatment of trypanosomiasis (sleeping sickness) in Africa, both in adults and children\textsuperscript{5,6}. The drug has also been shown to have antiviral potency against a few viruses, including human immunodeficiency virus and Rift Valley fever virus\textsuperscript{7,8}. It is, however, toxic at high doses and great care must be taken when administrated to children, especially infants\textsuperscript{9,10}.

The findings reported in the present study demonstrated that suramin can act in synergy with competitive inhibitors of hPIV-3 HN to block infection \textit{in vitro}. In particular, such an effect was observed with the anti-influenza virus drug zanamivir. Because they are synergistic, lower quantities of drugs are needed to reach a potent antiviral effect. This offers great potential for combinatorial therapy, especially given the fact that both molecules are approved drugs. The efficacy of suramin, however, has not been tested \textit{in vivo} for hPIV-3 infection, neither has the suramin–zanamivir combination. Such experiments would be crucial to determine whether non-toxic levels of suramin can be achieved \textit{in vivo} to block hPIV-3 infection, and whether the synergy with a competitive inhibitor would still occur in a systemic context.

\textit{In silico} docking analysis of a half suramin entity on hPIV-3 HN suggested that the molecule binds in close proximity to the active site. Given the high molecular weight of suramin and its high number of torsion angles (over 20), it was not possible to accurately predict the binding mode of the entire molecule. Therefore, additional experiments need to be undertaken to determine the exact mechanism of inhibition by suramin, namely to understand where it binds and if it induces a conformational change of the protein, reduces loop flexibility, or prevents ligands from transiting binding to the active site. Such experiments could involve resistance selection, epitope-mapping by reverse genetics, or co-crystallisation with a recombinant HN to obtain a suramin-bound structure.

Finally, since suramin is a molecule structurally unrelated to sialic acid, it provides a novel scaffold for the development of sialidase inhibitors. The drug most-likely binds HN via its sulfonate moieties, but its large size may prevent it from easily accessing positively-charged regions. One could thus envisage screening for simple fragments based on \textit{s}-naphtalenetrisulfonate framework, the building block of suramin, and expanding it to obtain more potent molecules.
Cyclopamine, a potent inhibitor of hRSV M2-1-dependent replication complex

In the third study, the identification, by phenotype-based screening, of the sonic hedgehog signalling pathway antagonist cyclopamine as a potent inhibitor of hRSV infection \textit{in vitro} and \textit{in vivo} has been presented (Section 3.2, p. 134). The compound was shown to block late events of hRSV replication, by acting independently of the aforementioned pathway. As a result, a target deconvolution by resistance selection was undertaken in an attempt to identify a viral partner of CPM. M2-1, the co-factor of the hRSV RdRp, was hypothesised as a target for the compound. By conducting minigenome experiments in presence of CPM, it was demonstrated that the molecule potently inhibits the function of the M2-1-dependent replication complex specifically. Moreover, CPM was shown to be able to block hRSV infection in a mouse model.

Cyclopamine is the first reported inhibitor of M2-1-dependent hRSV transcription. A few molecules have been shown to inhibit hRSV RdRp \textit{in vitro}, but no involvement with M2-1 has ever been described\textsuperscript{11–13}. Aside from blocking the function of the replication complex in a potent manner, we have found in this study that CPM altered cytoplasmic expression levels of M2-1. The reason for this diminished expression profile remains unknown, but is probably due to a destabilisation or precipitation of the protein. It is unlikely that the sole transcription of the M2-1 mRNA could be affected, since the transcription of hRSV genome is linear from the 5’ to the 3’ end and the expression of other proteins of the complex have not been altered.

Despite these observations, the reduction of cytoplasmic levels of M2-1 does not seem important enough to explain the inhibition of the replication complex by CPM on its own. All of the data from biochemical assays that were used to evaluate the effect of CPM on M2-1 binding to P or RNA were negative, however they were performed using un-complexed proteins. Similarly, CPM did not bind M2-1 in solution-based NMR experiments. It is therefore possible that the influence of CPM in this system may only be observed when the proteins are in complex. However, given the number of partners, 5 including RNA (N, P, L, M2-1), such experiments would be difficult to implement. Minigenome assays in the presence of a fluorescent-labelled CPM, however, could be an
option to colocalise the compound with a replication complex partner.

The principal mutated M2-1 amino acid that confers virus resistance to CPM (R151K) is located in the vicinity of 2 serines, at positions 58 and 61, whose state of phosphorylation has been shown to be critical for the anti-termination activity of the protein\textsuperscript{14,15}. Whether CPM has an indirect effect on the phosphorylation state of M2-1 is another hypothesis. However, phosphorylation is a transient process, and both phosphorylated and un-phosphorylated forms of M2-1 are needed for the virus to replicate efficiently\textsuperscript{14,16}. Such a theory could possibly be verified by minigenome experiments in the presence of CPM, by selectively substituting one serine or the other by the phosphoserine-mimetic aspartic acid, as it has been reported in previous studies\textsuperscript{14,17}.

As a known inhibitor of the Sonic hedgehog signalling pathway, a pathway involved in embryo development among others, the future of CPM as a treatment for paediatric infections maybe limited. Nevertheless, its discovery as a potent inhibitor of hRSV infection \textit{in vitro} and \textit{in vivo} has shed light on novel mechanisms of hRSV inhibition. CPM provides an unexplored scaffold for the discovery of potent inhibitors of the replication complex, and efforts should therefore be made towards the synthesis and screening of CPM derivatives that do not have an effect on the Shhp.

**Suramin, an inhibitor of EV71 infection \textit{in vitro} and \textit{in vivo}**

Finally, the fourth study described the discovery using a phenotypic screening approach that the trypanocidal drug suramin is an inhibitor of EV71 infection \textit{in vitro} and \textit{in vivo} (Section 4.2, p. 173). The drug, a poly-sulfonated aromatic compound, was characterised as an entry inhibitor of EV71 infection, acting by direct binding of the drug sulfonate groups to the virus capsid. The research was quickly advanced to pre-clinical studies where PK, PD and \textit{in vivo} efficacy were assessed. The drug was found to be protective against EV71 infection in a rhesus monkey model.

Considering the pressing need to develop drugs against EV71 infection and HFMD, it was thought that a phenotypic screening of approved drugs would be the most efficient way of identifying potential candidates, that could then be repositioned for HFMD treatment. As discussed in this Thesis, several compounds have been reported to inhibit EV71
Chapter 5. Conclusion and Future Directions

infection in vitro, notably rupintrivir and pleconaril (Section 1.4.3.2, p. 50). Rupintrivir, an experimental treatment for rhinovirus infections, has been discontinued after beginning phase II/III clinical trials. Pleconaril is also under development for the treatment of rhinovirus infection, but no further advances have been reported since it reached phase II clinical trials.

It was previously mentioned that suramin, although approved in children as well as in adults, can be toxic at high doses. Fortunately, in monkeys only an eleventh of the equivalent highest recommended human dose is required to block EV71 infection, which corresponds to more than 10-times the in vitro IC₉₀ of the drug. Therefore, given its status suramin makes a very good candidate for further advancement to clinical trials, especially for severe cases of infection.

Evidence was provided in this study that demonstrate that suramin binds the virus capsid thanks to its sufonated groups. Moreover, the crystal structure of EV71 capsid has been recently solved in its apo-form and in complex with various inhibitors¹⁸,¹⁹. Since the capsid has been co-crystallised, it would be worthwhile attempting to perform similar experiments in the presence of suramin so that a precise binding mechanism may be characterised. Meanwhile, in silico docking and molecular dynamics simulations could be carried out in an attempt to predict a binding site of the drug on the capsid, so that novel sulfated and sulfonated inhibitors could be rationally developed.

Concluding remarks

The work presented in this Thesis is oriented towards the discovery of novel drug candidates of paediatric viral infections, and provided invaluable insight into the development of novel inhibitors of human parainfluenza type-3 virus, human respiratory syncytial virus, and enterovirus 71. Four antiviral compounds were discovered, all possessing very different properties, and were identified using several drug discovery approaches. Rational and empirical methods were employed with both methods leading to conclusive results. It is expected that future research will build upon the present discoveries, to further increase the chance of paediatric antivirals to make it to the clinic.
References


Appendix 1

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References

Appendix 2

Supplementary material to
Guillon et al., 2014

This appendix contains supplementary material related to the paper:

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Reagents and Conditions: (i) (Boc)₂O, DMAP, THF, 60 °C, o/n, (18, 71%; 19, 96%) (ii) NaOCH₃/CH₃OH, rt, 1h; (iii) Ac₂O, pyridine, rt, o/n, (20, 81%; 21, 63% over 2 steps) (iv) TFA, DCM, rt, o/n, (22, 90%; 23, 85%) (v) Isobutyryl chloride, Et₃N, DCM, rt, 4h, (24, 84%; 12, 91%) (vi) NaOH, MeOH/H₂O (1:1), rt, o/n, (5, 94%; 6, 82%).

Supplementary Figure 2. Reagents and Conditions: (i) CuSO₄, Na-ascorbate, tert-butanol/H₂O (1:1), 45 °C, 6 h (13, 78%; 14, 82%; 15, 71%; 16, 84%); (ii) NaOH, MeOH/H₂O (1:1), rt, o/n (7, 85%; 8, 96%; 9, 92%; 10, 89%).
Supplementary Figure 3. $^1$H and $^{13}$C NMR spectra of reference and novel inhibitors.

a $^1$H NMR spectrum of 2 (300 MHz, D$_2$O)

$^{13}$C NMR spectrum of 2 (75 MHz, D$_2$O)
b $^1$H NMR spectrum of 3 (300 MHz, D$_2$O)

$^{13}$C NMR spectrum of 3 (75 MHz, D$_2$O)

Appendix 2.
C $^{1}$H NMR spectrum of 5 (300 MHz, D$_2$O)

$^{13}$C NMR spectrum of 5 (75 MHz, D$_2$O)
$^1$H NMR spectrum of 6 (300 MHz, D$_2$O)

$^{13}$C NMR spectrum of 6 (75 MHz, D$_2$O)
e $^1$H NMR spectrum of 7 (300 MHz, D$_2$O)

$^{13}$C NMR spectrum of 7 (75 MHz, D$_2$O)
$^1$H NMR spectrum of 8 (300 MHz, D$_2$O)

$^{13}$C NMR spectrum of 8 (75 MHz, D$_2$O)
$^1$H NMR spectrum of 9 (300 MHz, D$_2$O)

$^{13}$C NMR spectrum of 9 (75 MHz, D$_2$O)
$^1$H NMR spectrum of 10 (300 MHz, D$_2$O)

$^{13}$C NMR spectrum of 10 (75 MHz, D$_2$O)
**i **$^1$H NMR spectrum of 12 (300 MHz, CDCl$_3$)

$^{13}$C NMR spectrum of 12 (75 MHz, CDCl$_3$)

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Supplementary material to Guillon et al., 2014
$^1$H NMR spectrum of 13 (300 MHz, CDCl$_3$)

$^{13}$C NMR spectrum of 13 (75 MHz, CDCl$_3$)
**$^1$H NMR spectrum of 14 (300 MHz, CDCl$_3$)**

**$^{13}$C NMR spectrum of 14 (75 MHz, CDCl$_3$)**
$^1$H NMR spectrum of 15 (300 MHz, CDCl₃)

$^{13}$C NMR spectrum of 15 (75 MHz, CDCl₃)
\( ^1 \text{H NMR spectrum of 18 (300 MHz, CDCl}_3 \) 

\[ \text{H3, H4, H5, H6, H7, H8, H9, H9'} \]

\( ^{13} \text{C NMR spectrum of 18 (75 MHz, d}_6\text{-DMSO) } \)

\[ \text{NAc, 4OAc, 1-Boc-3CH}_3 \]
\[1^1H\) NMR spectrum of 19 (300 MHz, \(d_6\)-DMSO)

\[\text{13C NMR spectrum of 19 (75 MHz, } d_6\text{-DMSO)}\]
$^1$H NMR spectrum of 20 (300 MHz, CDCl$_3$)

$^{13}$C NMR spectrum of 20 (75 MHz, CDCl$_3$)

Appendix 2.
$^1$H NMR spectrum of 21 (300 MHz, CDCl₃)

$^{13}$C NMR spectrum of 21 (75 MHz, CDCl₃)
$^1$H NMR spectrum of 22 (300 MHz, CDCl$_3$)

$^{13}$C NMR spectrum of 22 (75 MHz, d$_6$-DMSO)
**S** $^1$H NMR spectrum of 23 (300 MHz, CD$_3$CN)

**$^{13}$C NMR spectrum of 23 (75 MHz, CD$_3$CN)**

Supplementary material to Guillon et al., 2014
\( ^{1}H \) NMR spectrum of 24 (300 MHz, CDCl\(_{3}\))

\( ^{13}C \) NMR spectrum of 24 (75 MHz, CDCl\(_{3}\))
Supplementary Figure 4. Comparison of NI (solid bar) and HI (dashed bar) IC₅₀ values for selected inhibitors. (a) Comparison of NI and HI IC₅₀ values for compounds 3, 6-10 and their C-4 hydroxyl analogues (2 and 5). (b) Comparison of NI and HI IC₅₀ values for compounds 7-10 and their C-4 azido analogues (3 and 6). (c) Comparison of NI and HI IC₅₀ values for compounds 6, 9 and 10 and their C-5 acetamido analogues (3, 7 and 8 respectively). The NI and HI results are representative of 3 independent experiments performed in triplicate or duplicate, respectively. The error bars correspond to the calculated standard deviation (calculated with GraphPadPrism4 (GraphPad Software Inc., La Jolla, CA).
Supplementary Figure 5. Comparison of HI IC\textsubscript{50} values for inhibitors 6 and 10, using guinea pig red blood cells (gp, RBC solid bar) and human red blood cells (h, RBC dashed bar). These results are representative of 3 independent experiments performed in duplicate and error bars correspond to the standard deviation (calculated with GraphPadPrism4 (GraphPad Software Inc., La Jolla, CA).
**Supplementary Figure 6. Evaluation of inhibitors in cell-based assays.** (a) Titration (focus forming assay) of progeny virus after a 48 h virus growth inhibition assay. Representative results of a progeny virus titration. Virus was harvested after 48 h amplification in the presence of 2 µM of compounds 8, 10 or 6. Collected virus-culture supernatants were diluted at least 1:1000 to make sure the remaining compound has no effect on foci formation. (b) Virus growth inhibition of the reference inhibitor 6 and inhibitor 10. Virus growth inhibition was determined by titration of progeny from a low MOI infected confluent LLC-MK2 monolayer in the presence of 2 µM inhibitor. At this inhibitor concentration, 10 showed 94% inhibition compared with 14% inhibition for 6. These results are representative of 2 independent experiments performed in duplicate and error bars correspond to the standard deviation (calculated with GraphPadPrism4 (GraphPad Software Inc., La Jolla, CA).
Supplementary Figure 7. ^1^H and STD NMR spectra of 8 in complex with hPIV-3 HN. (a) ^1^H NMR spectrum of 8 and (b) STD NMR spectrum of 8 in the presence of 20 μM hPIV-3 HN at a protein-ligand ratio of 1:100 (2 mM 8). (c) ^1^H NMR spectrum of the H7, H8, H9 and H9’ region. Signals from residual glycerol are marked as □ (d) STD NMR spectrum of the H7, H8, H9 and H9’ region. (e) The proposed binding epitope of 8.
Supplementary Figure 8. STD NMR spectra comparison of 10 in complex with intact hPIV-3 virus or recombinant HN. (a) $^1$H NMR spectrum of 10 in the presence of hPIV-3 HN, (b) STD NMR spectrum of 10 in the presence of intact hPIV-3 virus and (c) STD NMR spectrum of 10 in the presence of hPIV-3 HN.

Supplementary Figure 9. Superimposition of the phenyl protons from 10 in complex with intact virus or recombinant HN. (a) $^1$H NMR spectrum of 10 and (b) Superimpositions of STD NMR spectra from 10 in the presence of hPIV-3 virus (black) or recombinant hPIV-3 HN (red).
SUPPLEMENTARY TABLES

**Supplementary Table 1. Molecular Dynamics Simulations Parameters for 8.**

![Molecular structure of 8](image_url)

**a** Non-bonded parameters

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Supplementary Table 2. NI and HI IC$_{50}$ values. Mean IC$_{50}$ values for each of the tested compounds are presented, together with calculated standard deviation and standard error.

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Supplementary Table 3. Relative STD NMR effects* of 8 and 10 in complex with hPIV-3 HN.

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<td>36</td>
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<tr>
<td>H8</td>
<td>30</td>
<td>35</td>
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<tr>
<td>H9</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>H9’</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td>Isoprop-CH</td>
<td>-</td>
<td>54</td>
</tr>
<tr>
<td>Isoprop-2CH$_3$</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>NHAc</td>
<td>41</td>
<td>-</td>
</tr>
</tbody>
</table>

*STD effects were calculated according to the formula $A_{STD} = (I_0 - I_{sat}) / I_0 - I_{STD} / I_0$. All STD NMR effects are given relative to the strongest STD NMR intensity of the C4 triazolo ArH4’.
SUPPLEMENTARY METHODS

General Chemistry Methods: Reagents and dry solvents were purchased from commercial sources and used without further purification. Anhydrous reactions were carried out under an atmosphere of argon in oven-dried glassware. Reactions were monitored using thin layer chromatography (TLC) on aluminium plates pre-coated with Silica Gel 60 F254 (E. Merck). Developed plates were observed under UV light at 254 nm and then visualized after application of a solution of H₂SO₄ in EtOH (5% v/v) followed by charring. Flash chromatography was performed on Silica Gel 60 (0.04 - 0.063 mm) using distilled solvents. ¹H and ¹³C NMR spectra (Supplementary Fig. 3a-t) were recorded at 300 and 75.5 MHz respectively on a BrukerAvance 300 MHz spectrometer. Chemical shifts (δ) are reported in parts per million, relative to the residual solvent peak as internal reference [CDCl₃]: 7.26 (s) for ¹H, 77.0 (t) for ¹³C; DMSO: 2.50 (pent) for ¹H, 39.51 (hept) for ¹³C; D₂O: 4.79 (s) for ¹H]. 2D COSY and HSQC experiments were run to support assignments. Low-resolution mass spectra (LRMS) were recorded, in electrospray ionization mode, on a BrukerDaltonics Esquire 3000 ESI spectrometer, using positive mode. High-resolution mass spectrometry (HRMS) were recorded for either the protected or deprotected final derivatives, and were carried out by the University of Queensland FTMS Facility on a BrukerDaltonics Apex III 4.7e Fourier Transform micrOTOF-Q70 MS or by the Griffith University FTMS Facility on a BrukerDaltonics Apex III 4.7e Fourier Transform MS, fitted with an Apollo ESI source.

Final deprotected sialic acid derivatives were purified on a GracePure™ SPE C18-Aq (5000 mg/20 mL) column using 2% acetonitrile/H₂O as a solvent. The purity of all synthetic intermediates after chromatographic purification was determined to be >90% by ¹H and ¹³C NMR spectroscopy and the purity of reference compounds synthesised for screening purposes (2, 3, 5, 6), as well as the novel final products 7-10, was determined to be ≥95%.
Characterisation of Synthesised Inhibitors

Sodium 5-acetamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonate (Neu5Ac2en, 2)\(^1\).

\[
\text{Neu5Ac2en (2)}
\]

\(^1\)H NMR (300 MHz, D\(_2\)O): \(\delta\) 2.08 (s, 3H, NAc), 3.54–3.74 (m, 2H, H-7, H-9), 3.84–4.00 (m, 2H, H-8, H-9\(^{-}\)), 4.07 (dd, \(J = 10.9, 8.7\) Hz, 1H, H-5), 4.23 (d, \(J = 10.9\) Hz, 1H, H-6), 4.48 (dd, \(J = 8.7, 2.3\) Hz, 1H, H-4), 5.70 (d, \(J = 2.4\) Hz, 1H, H-3); \(^{13}\)C NMR (75 MHz, D\(_2\)O): \(\delta\) 22.08 (NHCOCH\(_3\)), 49.84 (C-5), 63.10 (C-9), 67.56 (C-4), 68.21 (C-7), 69.73 (C-8), 75.29 (C-6), 107.53 (C-3), 147.92 (C-2), 169.62 (COONa), 174.68 (NHCOCH\(_3\)); LRMS \([\text{C}_{11}\text{H}_{16}\text{NNaO}_{8}]\) (m/z): (+ve ion mode) 335.9 [M+Na]\(^+\).

Sodium 5-acetamido-2,6-anhydro-4-azido-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonate (3)\(^2\).

\[
\text{Neu5Ac2en (3)}
\]

\(^1\)H NMR (300 MHz, D\(_2\)O): \(\delta\) 2.09 (s, 3H, NAc), 3.63–3.69 (m, 2H, H-7, H-9), 3.82–4.04 (m, 2H, H-8, H-9\(^{-}\)), 4.15–4.43 (m, 3H, H-4, H-5, H-6), 5.72 (d, \(J = 2.2\) Hz, 1H, H-3); \(^{13}\)C NMR (75 MHz, D\(_2\)O): \(\delta\) 22.09 (NHCOCH\(_3\)), 47.72 (C-5), 59.24 (C-4), 63.07 (C-9), 68.02 (C-7), 69.73 (C-8), 75.21 (C-6), 103.32 (C-3), 149.37 (C-2), 169.07 (COONa), 174.51 (NHCOCH\(_3\)); LRMS \([\text{C}_{11}\text{H}_{15}\text{NNaO}_{7}]\) (m/z): (+ve ion mode) 360.9 [M+Na]\(^+\).
Methyl 5-[Acetyl-(tert-butoxycarbonyl)amino]-4,7,8,9-tetra-O-acetyl-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonate (18)\(^1\).

\[\text{Diagram of 18}\]

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 1.55 (s, 9H, t-Boc-3CH\(_3\)), 1.97 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.35 (s, 3H, NAc), 3.77 (s, 3H, COOCH\(_3\)), 4.12 (dd, \(J = 12.4\), 6.0 Hz, 1H, H-9), 4.59 (d, \(J = 12.7\) Hz, 1H, H-9'), 4.75–5.40 (m, 4H, H-5, H-6, H-7, H-8), 5.89 (d, \(J = 2.7\) Hz, 1H, H-3), 6.02 (d, \(J = 7.9\) Hz, 1H, H-4); \(^{13}\)C NMR (75 MHz, \(d_6\)-DMSO): \(\delta\) 20.32, 20.38, 20.41, 20.45 (4 OCOCH\(_3\)), 27.28 (t-Boc-3CH\(_3\)), 49.99 (C-5), 52.42 (COOCH\(_3\)), 61.16 (C-9), 66.00 (C-4), 66.88 (C-7), 69.66 (C-8), 76.22 (C-6), 84.85 (t-Boc-\(\_\_\_\_\_\)_CH\(_3\)), 109.34 (C-3), 145.65 (C-2), 154.37 (t-Boc-OCO), 161.05 (COOCH\(_3\)), 169.29, 169.63, 170.06, 170.19 (4 OCOCH\(_3\), CH\(_3\)CONH); LRMS \([C_{23}H_{32}N_4O_{12}] (m/z): (+ve ion mode) 596.2 [M+Na]^{+}\).

Methyl 5-[Acetyl-(tert-butoxycarbonyl)amino]-7,8,9-tri-O-acetyl-2,6-anhydro-4-azido-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonate (19)\(^1\).

\[\text{Diagram of 19}\]

\(^1\)H NMR (300 MHz, \(d_6\)-DMSO): \(\delta\) 1.52 (s, 9H, t-Boc-3CH\(_3\)), 1.96 (s, 6H, 2OAc), 1.97 (s, 3H, OAc), 2.33 (s, 3H, NAc), 3.72 (s, 3H, COOCH\(_3\)), 4.05 (m, 1H, H-9), 4.47 (m, 1H, H-9'), 4.63–4.91 (m, 3H, H-4, H-5, H-6), 5.11–5.38 (m, 2H, H-7, H-8), 5.98 (d, \(J = 2.4\) Hz, 1H, H-3); \(^{13}\)C NMR (75 MHz, \(d_6\)-DMSO): \(\delta\) 20.36, 20.42 (3 OCOCH\(_3\), NCOCH\(_3\)), 27.20 (t-Boc-3CH\(_3\)), 50.32 (C-5), 52.47 (COOCH\(_3\)), 56.93 (C-4), 61.08 (C-9), 65.92 (C-7), 69.56 (C-8), 76.54 (C-6), 85.44 (t-Boc-\(\_\_\_\_\_\)_CH\(_3\)), 108.64 (C-3), 145.51 (C-2), 151.18 (t-Boc-OCO), 161.00 (COOCH\(_3\)), 169.24, 169.69, 170.05 (3 OCOCH\(_3\), 172.71 (CH\(_3\)CONH); LRMS \([C_{23}H_{32}N_4O_{12}] (m/z): (+ve ion mode) 579.3 [M+Na]^{+}\).
Supplementary material to Guillon et al., 2014

Methyl 4,7,8,9-tetra-O-acetyl-2,6-anhydro-5-(tert-butoxycarboxamido)-3,5-dideoxy-D-glycero-D-galacto-non-2-enate (20)\(^3\).

![Structural diagram of Compound 20](image)

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 1.39 (s, 9H, t-Boc-3CH\(_3\)), 2.04 (s, 6H, 2 OAc), 2.06 (s, 3H, OAc), 2.11 (s, 3H, OAc), 3.78 (s, 3H, COOCH\(_3\)), 4.01–4.12 (m, 1H, H-5), 4.17 (dd, \(J = 12.2, 6.8\) Hz, 1H, H-9), 4.32 (dd, \(J = 9.0, 3.7\) Hz, 1H, H-6), 4.58 (dd, \(J = 12.3, 3.4\) Hz, 1H, H-9\(^\prime\)), 4.65 (d, \(J = 9.8\) Hz, 1H, NH), 5.35 (ddd, \(J = 6.8, 5.2, 3.5\) Hz, 1H, H-8), 5.46 (dd, \(J = 7.5, 3.1\) Hz, 1H, H-4), 5.53 (t, \(J = 4.2\) Hz, 1H, H-7), 5.97 (d, \(J = 3.0\) Hz, 1H, H-3); \(^13\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 20.67, 20.71, 20.78, 20.83 (4 OCH\(_3\)), 28.14 (t-Boc-3CH\(_3\)), 47.88 (C-5), 52.53 (COOCH\(_3\)), 61.96 (C-9), 67.79 (C-7), 68.46 (C-4), 70.45 (C-8), 76.80 (C-6), 80.45 (t-Boc-CH\(_3\)), 107.97 (C-3), 145.01 (C-2), 154.84 (t-Boc-OCO), 161.64 (COOCH\(_3\)), 169.78, 169.93, 170.56, 170.60 (4 OCH\(_3\)); LRMS [C\(_{21}\)H\(_{30}\)N\(_4\)O\(_{11}\)] (m/z): (+ve ion mode) 554.2 [M+Na\(^+\)].

Methyl 7,8,9-tri-O-acetyl-2,6-anhydro-4-azido-5-(tert-butoxycarboxamido)-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enate (21)\(^3\).

![Structural diagram of Compound 21](image)

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 1.43 (s, 9H, t-Boc-3CH\(_3\)), 2.05 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.13 (s, 3H, OAc), 3.68 (q, \(J = 9.3\) Hz, 1H, H-5), 3.80 (s, 3H, COOCH\(_3\)), 4.17 (dd, \(J = 12.4, 6.7\) Hz, 1H, H-9), 4.28–4.42 (m, 2H, H-4, NH), 4.62 (dd, \(J = 12.4, 2.9\) Hz, 1H, H-9\(^\prime\)), 4.81 (d, \(J = 9.3\) Hz, 1H, H-6), 5.33 (ddd, \(J = 6.7, 5.3, 2.9\) Hz, 1H, H-8), 5.50 (dd, \(J = 5.4, 2.5\) Hz, 1H, H-7), 5.95 (d, \(J = 2.7\) Hz, 1H, H-3); \(^13\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 21.27, 21.41 (3 OCH\(_3\)), 28.76 (t-Boc-3CH\(_3\)), 49.80 (C-5), 53.11 (COOCH\(_3\)), 58.94 (C-4), 62.58 (C-9), 68.31 (C-7), 71.31 (C-8), 76.74 (C-6), 81.36 (t-Boc-CH\(_3\)), 108.18 (C-3), 145.64 (C-2), 155.28 (t-Boc-OCO), 162.08 (COOCH\(_3\)), 170.56, 170.61, 171.15 (3 OCH\(_3\)); LRMS [C\(_{23}\)H\(_{30}\)N\(_4\)O\(_{11}\)] (m/z): (+ve ion mode) 537.3 [M+Na\(^+\)].
Methyl 4,7,8,9-tetra-O-acetyl-5-amino-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonate (22)\(^3\).

Silica gel column chromatography was run using ethyl acetate:hexane (5:1). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta 1.65\) (s, 2H, NH\(_2\)), 2.03 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.14 (s, 3H, OAc), 2.95 (t, \(J = 9.0\) Hz, 1H, H-5), 3.76 (s, 3H, COOCH\(_3\)), 3.95 (d, \(J = 9.8\) Hz, 1H, H-6), 4.24 (dd, \(J = 12.6, 5.2\) Hz, 1H, H-9), 4.54 (dd, \(J = 12.3, 2.5\) Hz, 1H, H-9'), 5.27 (dd, \(J = 8.0, 2.7\) Hz, 1H, H-4), 5.42 (m, 1H, H-8), 5.55 (d, \(J = 6.4\) Hz, 1H, H-7), 5.90 (s, 1H, H-3); \(^{13}\)C NMR (75 MHz, \(d_6\)-DMSO): \(\delta 25.65, 25.69, 25.77, 26.02\) (4 OCOCH\(_3\)), 52.82 (C-5), 57.52 (COOCH\(_3\)), 66.82 (C-9), 72.76 (C-7), 74.40 (C-4), 76.37 (C-8), 82.98 (C-6), 113.50 (C-3), 149.37 (C-2), 166.70 (COOCH\(_3\)), 174.68, 175.35, 175.40, 175.84 (3 OCOCH\(_3\)); LRMS \([\text{C}_{18}\text{H}_{25}\text{NO}_{11}]\) (m/z): (+ve ion mode) 454.1 [M+Na].

Methyl 7,8,9-tri-O-acetyl-5-amino-2,6-anhydro-4-azido-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonate (23)\(^3\).

Silica gel column chromatography was run using ethyl acetate:hexane (2:1). \(^1\)H NMR (300 MHz, CD\(_3\)CN): \(\delta 1.99\) (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.71 (t, \(J = 9.5\) Hz, 1H, H-5), 3.74 (s, 3H, COOCH\(_3\)), 3.94–4.02 (m, 2H, H-4, H-6), 4.19 (dd, \(J = 12.5, 5.5\) Hz, 1H, H-9), 4.45 (dd, \(J = 12.6, 2.7\) Hz, 1H, H-9'), 5.31 (dd, \(J = 7.8, 5.4, 2.7\) Hz, 1H, H-8), 5.57 (d, \(J = 7.3\) Hz, 1H, H-7), 5.87 (d, \(J = 2.5\) Hz, 1H, H-3); \(^{13}\)C NMR (75 MHz, CD\(_3\)CN): \(\delta 21.28, 21.37, 21.47\) (3 OCOCH\(_3\)), 50.84 (C-5), 53.39 (COOCH\(_3\)), 62.53 (C-4), 63.18 (C-9), 69.03 (C-7), 70.68 (C-8), 79.72 (C-6), 109.41 (C-3), 146.29 (C-2), 163.16 (COOCH\(_3\)), 171.03, 171.76, 171.97 (3 OCOCH\(_3\)); LRMS \([\text{C}_{16}\text{H}_{22}\text{N}_{4}\text{O}_{9}]\) (m/z): (+ve ion mode) 437.1 [M+Na].
Methyl 4,7,8,9-tetra-O-acetyl-2,6-anhydro-3,5-dideoxy-5-isobutyramido-D-glycero-D-galacto-non-2-enonate (24).\(^1\)

\[
\text{Methyl 4,7,8,9-tetra-O-acetyl-2,6-anhydro-3,5-dideoxy-5-isobutyramido-D-glycero-D-galacto-non-2-enonate (24).}
\]

\[\text{H NMR (300 MHz, CDCl}_3\text{): } \delta 1.10 (d, J = 6.8 Hz, 6H, isobutyryl-2CH}_3\text{), 2.04 (s, 3H, OAc), 2.06 (s, 6H, 2OAc), 2.12 (s, 3H, OAc), 2.27 (m, 1H, isobutyryl-CH), 3.80 (s, 3H, COOCH}_3\text{), 4.19 (dd, J = 12.3, 6.9 Hz, 1H, H-9), 4.30–4.47 (m, 2H, H-5, H-6), 4.60 (dd, J = 12.3, 3.2 Hz, 1H, H-9\text{'), 5.33 (m, 1H, H-8), 5.41–5.52 (m, 2H, H-7, NH), 5.57 (dd, J = 7.2, 3.1 Hz, 1H, H-4), 5.99 (d, J = 3.0 Hz, 1H, H-3); ^1^C NMR (75 MHz, CDCl}_3\text{): } \delta 18.92, 19.32 (isobutyryl-2CH}_3\text{), 20.67, 20.70, 20.76, 20.83 (4 COOCH}_3\text{), 35.59 (isobutyryl-CH), 46.34 (C-5), 52.53 (COOCH}_3\text{), 61.96 (C-9), 67.61 (C-7), 68.05 (C-4), 71.04 (C-8), 76.68 (C-6), 108.11 (C-3), 145.09 (C-2), 161.60 (COOCH}_3\text{), 170.02, 170.31, 170.54, 170.80 (4 COOCH}_3\text{), 176.94 (isobutyryl-CO); LRMS [C_{20}H_{32}N_{11}O_{11}] (m/z): (+ve ion mode) 524.3 [M+Na]^+\].

Methyl 7,8,9-tri-O-acetyl-2,6-anhydro-4-azido-3,4,5-trideoxy-5-isobutyramido-D-glycero-D-galacto-non-2-enonate (12).\(^1\)

\[
\text{Methyl 7,8,9-tri-O-acetyl-2,6-anhydro-4-azido-3,4,5-trideoxy-5-isobutyramido-D-glycero-D-galacto-non-2-enonate (12).}
\]

\[\text{H NMR (300 MHz, CDCl}_3\text{): } \delta 1.09–1.18 (m, 6H, isobutyryl-2CH}_3\text{), 2.01 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.34 (m, 1H, isobutyryl-CH), 3.61–3.85 (m, 4H, COOCH}_3\text{, 5.47 (dd, J = 12.5, 6.3 Hz, 1H, H-9), 4.50–4.69 (m, 3H, H-4, H-6, H-9\text{'), 5.27 (m, 1H, H-8), 5.38 (dd, J = 5.6, 2.1 Hz, 1H, H-7), 5.92 (d, J = 2.6 Hz, 1H, H-3), 6.04 (d, J = 8.4 Hz, 1H, NH); ^1^C NMR (75 MHz, CDCl}_3\text{): } \delta 18.95, 19.32 (isobutyryl-2CH}_3\text{), 20.67, 20.73, 20.81 (3 COOCH}_3\text{), 35.74 (isobutyryl-CH), 49.14 (C-5), 52.53 (COOCH}_3\text{), 57.33 (C-4), 61.91 (C-9), 67.68 (C-7), 70.63 (C-8), 75.29 (C-6), 107.73 (C-3), 145.03 (C-2), 161.57 (COOCH}_3\text{), 170.06, 170.35, 170.58 (3 COOCH}_3\text{), 177.59 (isobutyryl-CO); LRMS [C_{20}H_{32}N_{11}O_{11}] (m/z): (+ve ion mode) 507.1 [M+Na]^+\].
Sodium 2,6-anhydro-3,5-dideoxy-5-isobutyramido-D-glycero-D-galacto-non-2-enonate (5)^3.

\[
\text{5}
\]

\(^1\)H NMR (300 MHz, D\textsubscript{2}O): \(\delta 1.14–1.17\) (m, 6H, isobutyryl-2CH\textsubscript{3}), 2.59 (m, 1H, isobutyryl-CH), 3.57 (dd, \(J = 9.3, 1.2\) Hz, 1H, H-7), 3.65 (dd, \(J = 11.8, 6.1\) Hz, 1H, H-9), 3.85–4.00 (m, 2H, H-8, H-9\(^\prime\)), 4.05 (dd, J = 10.9, 8.8 Hz, 1H, H-5), 4.25 (dd, J = 11.1, 1.2 Hz, 1H, H-6), 4.50 (dd, J = 8.8, 2.4 Hz, 1H, H-4), 5.71 (d, \(J = 2.3\) Hz, 1H, H-3); \(^1\)C NMR (75 MHz, D\textsubscript{2}O): \(\delta 18.46, 18.91\) (isobutyryl-2CH\textsubscript{3}), 35.22 (isobutyryl-CH), 49.57 (C-5), 63.08 (C-9), 67.35 (C-4), 68.29 (C-7), 69.73 (C-8), 75.31 (C-6), 107.72 (C-3), 147.87 (C-2), 169.65 (COONa), 181.78 (isobutyryl-CO); LRMS [C\textsubscript{13}H\textsubscript{20}NNaO\textsubscript{8}] (m/z): (+ve ion mode) 364.1 [M+Na\(^{+}\)].

Sodium 2,6-anhydro-4-azido-3,4,5-trideoxy-5-isobutyramido-D-glycero-D-galacto-non-2-enonate (6)^3.

\[
\text{BCX 2798 (6)}
\]

\(^1\)H NMR (300 MHz, D\textsubscript{2}O): \(\delta 1.14–1.17\) (m, 6H, isobutyryl-2CH\textsubscript{3}), 2.58 (p, \(J = 6.9\) Hz, 1H, isobutyryl-CH), 3.56–3.72 (m, 2H, H-7, H-9), 3.85–4.02 (m, 2H, H-8, H-9\(^\prime\)), 4.22 (dd, J = 10.7, 9.2 Hz, 1H, H-5), 4.29–4.41 (m, 2H, H-4, H-6), 5.71 (d, \(J = 2.3\) Hz, 1H, H-3); \(^1\)C NMR (75 MHz, D\textsubscript{2}O): \(\delta 18.45, 18.63\) (isobutyryl-2CH\textsubscript{3}), 35.28 (isobutyryl-CH), 47.54 (C-5), 59.30 (C-4), 63.05 (C-9), 68.10 (C-7), 69.75 (C-8), 75.16 (C-6), 103.44 (C-3), 149.31 (C-2), 169.09 (COONa), 181.47 (isobutyryl-CO); LRMS [C\textsubscript{13}H\textsubscript{19}N\textsubscript{4}O\textsubscript{7}] (m/z): (+ve ion mode) 367.2 [M+Na\(^{+}\)].
Methyl 5-acetamido-7,8,9-tri-O-acetyl-2,6-anhydro-3,4,5-trideoxy-4-(4-methoxymethyl-[1,2,3]triazol-1-yl)-D-glycero-D-galacto-non-2-enonate (13).

Purification by silica gel chromatography using ethyl acetate:acetone (6:1) yielded (90 mg, 78%) of pure 13.  

$^1$H NMR (300 MHz, CDCl$_3$): δ 1.81 (s, 3H, NAc), 2.05 (s, 6H, 2OAc), 2.06 (s, 3H, OAc), 3.36 (s, 3H, OCH$_3$), 3.80 (s, 3H, COOCH$_3$), 4.17 (dd, $J = 12.5, 7.2$ Hz, 1H, H-9), 4.29 (m, 1H, H-5), 4.50 (s, 2H, OCH$_2$), 4.68–4.79 (m, 2H, H-9', H-6), 5.40 (ddd, $J = 7.4, 4.9, 2.5$ Hz, 1H, H-8), 5.53 (dd, $J = 5.1, 1.8$ Hz, 1H, H-7), 5.78 (dd, $J = 10.0, 2.5$ Hz, 1H, H-4), 6.00 (d, $J = 2.3$ Hz, 1H, H-3), 7.05 (d, $J = 9.1$ Hz, 1H, NH), 7.64 (s, 1H, triazole-CH); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 20.71, 20.79, 20.91 (3 OCOCH$_3$), 22.80 (NHCOCH$_3$), 48.39 (C-5), 52.71 (COOCH$_3$)$_2$, 58.16 (OCH$_3$), 58.38 (C-4), 62.21 (C-9), 65.68 (OCH$_2$), 67.73 (C-7), 70.90 (C-8), 76.71 (C-6), 107.18 (C-3), 121.50 (triazole-C-5), 145.24 (triazole-C-4), 145.92 (C-2), 161.27 (COOCH$_3$), 170.06, 170.27, 170.81, 170.88 (NHCOCH$_3$, 3 OCOCH$_3$). LRMS [C$_{22}$H$_{30}$N$_4$O$_{11}$] (m/z): (+ve ion mode) 549.1 [M+Na]$^+$; HRMS (API) (m/z): [M+Na]$^+$ calcd for C$_{22}$H$_{30}$N$_4$NaO$_{11}$ [M+Na]$^+$ 549.1803; found, 549.1805.

Methyl 5-acetamido-7,8,9-tri-O-acetyl-2,6-anhydro-3,4,5-trideoxy-4-(4-phenyl-[1,2,3]triazol-1-yl)-D-glycero-D-galacto-non-2-enonate (14)$^\text{a}$.  

Purification by silica gel chromatography using ethyl acetate:hexane (5:1) yielded (103 mg, 82%) of pure 14.  

$^1$H NMR (300 MHz, CDCl$_3$): δ 1.79 (s, 3H, NAc), 2.04 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.06 (s, 3H, OAc), 3.81 (s, 3H, COOCH$_3$), 4.18 (dd, $J = 12.4, 7.2$ Hz, 1H, H-9), 4.42 (m, 1H, H-5), 4.72 (dd, $J = 12.6,
2.7 Hz, 1H, H-9´), 4.79 (dd, J = 10.5, 1.6 Hz, 1H, H-6), 5.42 (m, 1H, H-8), 5.58 (dd, J = 5.1, 1.8 Hz, 1H, H-7), 5.83 (dd, J = 10.0, 2.4 Hz, 1H, H-4), 6.04 (d, J = 2.3 Hz, 1H, H-3), 7.20–7.42 (m, 4H, NH, Ph-H-3´, Ph-H-4´, Ph-H-5´), 7.72 (dd, J = 8.2, 1.3 Hz, 2H, Ph-H-2´, Ph-H-6´), 7.88 (s, 1H, triazole-CH); 13C NMR (75 MHz, CDCl₃) δ 20.70, 20.80, 20.90 (3 OCOCH₃), 22.85 (NHCOCH₃), 48.26 (C-5), 52.71 (COOCH₃), 58.41 (C-4), 62.26 (C-9), 67.80 (C-7), 70.97 (C-8), 76.95 (C-6), 107.37 (C-3), 118.76 (triazole-C-5), 125.77 (Ph), 128.51 (Ph), 128.91 (Ph), 129.85 (Ph, q carbon), 145.95 (C-2), 148.13 (triazole-C-4), 161.32 (COCH₃), 170.04, 170.30, 170.84, 170.99 (NHOCCH₃, 3 OCOCCH₃). LRMS [C₂₆H₃₀N₄O₁₀] (m/z): (+ve ion mode) 581.0 [M+Na]⁺; HRMS (API) (m/z): [M+Na]⁺ calcd for C₂₆H₃₀N₄NaO₁₀ [M+Na]⁺ 581.185414; found, 581.184724.

Methyl 7,8,9-tri-O-acetyl-2,6-anhydro-3,4,5-trideoxy-5-isobutyramido-4-(4-methoxymethyl)[1,2,3]triazol-1-yl)-D-glycero-D-galacto-non-2-enonate (15).

Purification by silica gel chromatography using ethyl acetate:acetone (9:1) yielded (65 mg, 71%) of pure 15.

1H NMR (300 MHz, CDCl₃): 0.97 (d, J = 6.9 Hz, 3H, isobut-CH₃), 1.01 (d, J = 6.8 Hz, 3H, isobut-CH₃), 2.05 (s, 3H, OAc), 2.08 (s, 6H, 2OAc), 2.24 (m, 1H, isobut-CH), 3.37 (s, 3H, OCH₃), 3.81 (s, 3H, COOCH₃), 4.14–4.29 (m, 2H, H-9, H-5), 4.51 (s, 2H, OCH₂), 4.68 (dd, J = 12.5, 2.6 Hz, 1H, H-9´), 4.84 (dd, J = 10.5, 1.7 Hz, 1H, H-6), 5.38 (ddd, J = 6.6, 5.5, 2.5 Hz, 1H, H-8), 5.48 (dd, J = 5.5, 1.7 Hz, 1H, H-7), 5.91 (dd, J = 10.0, 2.4 Hz, 1H, H-4), 6.02 (d, J = 2.4 Hz, 1H, H-3), 6.44 (d, J = 8.7 Hz, 1H, NH), 7.59 (s, 1H, triazole-CH); 13C NMR (75 MHz, CDCl₃): δ 18.81, 19.30 (isobut-2CH₃), 20.74, 20.90 (3 OCOCH₃), 35.51 (isobut-CH), 48.79 (C-5), 52.69 (COOCH₃), 57.59 (C-4), 58.38 (OCH₃), 62.08 (C-9), 65.73 (OCH₂), 67.66 (C-7), 70.79 (C-8), 76.24 (C-6), 107.07 (C-3), 121.54 (triazole-C-5), 145.39 (triazole-C-4), 145.82 (C-2), 161.30 (COOCH₃), 170.15, 170.23, 170.69 (3 O COCH₃), 177.87 (isobut-CO). LRMS [C₂₅H₃₂N₄O₁₁] (m/z): (+ve ion mode) 577.2 [M+Na]⁺.
Methyl 7,8,9-tri-O-acetyl-2,6-anhydro-3,4,5-trideoxy-5-isobutyramido-4-(4-phenyl-[1,2,3]triazol-1-yl)-D-glycero-D-galacto-non-2-enonate (16).

Purification by silica gel chromatography using ethyl acetate:hexane (4:1) yielded (82 mg, 84%) of pure 16. 

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 0.95 (d, $J = 6.8$ Hz, 3H, isobut-CH$_3$), 0.99 (d, $J = 6.9$ Hz, 3H, isobut-CH$_3$), 2.06 (s, 3H, OAc), 2.09 (s, 6H, 2OAc), 2.20–2.27 (m, 1H, isobut-CH), 3.83 (s, 3H, COOCH$_3$), 4.16–4.39 (m, 2H, H-9, H-5), 4.70 (dd, $J = 12.5$, 2.6 Hz, 1H, H-9´), 4.88 (dd, $J = 10.5$, 1.7 Hz, 1H, H-6), 5.40 (m, 1H, H-8), 5.52 (dd, $J = 5.4$, 1.7 Hz, 1H, H-7), 5.99 (dd, $J = 10.0$, 2.4 Hz, 1H, H-4), 6.08 (d, $J = 2.4$ Hz, 1H, H-3), 6.51 (d, $J = 8.7$ Hz, 1H, NH), 7.26–7.43 (m, 3H, Ph-H-3´, Ph-H-4´, Ph-H-5´), 7.74 (d, $J = 7.2$ Hz, 2H, Ph-H-2´, Ph-H-6´), 7.81 (s, 1H, triazole-CH); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 18.83, 19.31 (isobut-2CH$_3$), 20.76, 20.92 (3 OCOCH$_3$), 35.55 (isobut-CH), 48.74 (C-5), 52.72 (COOCH$_3$), 57.67 (C-4), 62.12 (C-9), 67.72 (C-7), 70.84 (C-8), 76.39 (C-6), 107.25 (C-3), 118.84 (triazole-C-5), 125.83 (Ph), 128.47 (Ph), 128.89 (Ph), 129.97 (Ph q carbon), 145.81 (C-2), 148.19 (triazole-C-4), 161.35 (COOCH$_3$), 170.18, 170.26, 170.71 (3 OCOCH$_3$), 178.00 (isobut-CO). LRMS [C$_{28}$H$_{34}$N$_4$O$_{10}$] (m/z): (+ve ion mode) 608.9 [M+Na$^+$].

Sodium 5-acetamido-2,6-anhydro-3,4,5-trideoxy-4-(4-methoxymethyl-[1,2,3]triazol-1-yl)-D-glycero-D-galacto-non-2-enonate (7).

Yield = 85%. $^1$H NMR (300 MHz, D$_2$O): $\delta$ 1.84 (s, 3H, NAc), 3.31 (s, 3H, OCH$_3$), 3.52–3.71 (m, 2H, H-9 & H-7), 3.85 (dd, $J = 11.9$, 2.6 Hz, 1H, H-9´), 3.95 (ddd, $J = 9.3$, 6.2, 2.5 Hz, 1H, H-8), 4.33 (m, 1H, H-5), 5.39 (dd, $J = 10.6$, 1.7 Hz, 1H, H-6), 8.83 (s, 1H, triazole-CH).
4.51 (dd, J = 10.9, 1.2 Hz, 1H, H-6), 4.56 (s, 2H, OCH2), 5.48 (dd, J = 9.6, 2.3 Hz, 1H, H-4), 5.80 (d, J = 2.2 Hz, 1H, H-3), 8.08 (s, 1H, triazole-CH). 13C NMR (75 MHz, D2O): δ 21.65 (NHCOCH3), 48.68 (C-5), 57.15 (OCH3), 59.94 (C-4), 63.06 (C-9), 64.22 (OCH3), 68.05 (C-7), 69.71 (C-8), 75.34 (C-6), 101.80 (C-3), 123.54 (triazole-C-5), 144.08 (triazole-C-4), 150.43 (C-2), 168.75 (COONa), 173.57 (NHCOCH3); LRMS [C15H21N4NaO8] (m/z): (+ve ion mode) 432.1 [M+Na]+.

Sodium 5-acetamido-2,6-anhydro-3,4,5-trideoxy-4-(4-phenyl-[1,2,3]triazol-1-yl)-D-glycero-D-galacto-non-2-enonate (8).4

Yield = 96%. 1H NMR (300 MHz, D2O): δ 1.87 (s, 3H, NAc), 3.64 (dd, J = 12.1, 6.4 Hz, 1H, H-9), 3.69 (dd, J = 9.6, 1.4 Hz, 1H, H-7), 3.89 (dd, J = 11.9, 2.7 Hz, 1H, H-9'), 4.00 (ddd, J = 9.3, 6.3, 2.7 Hz, 1H, H-8), 4.39 (m, 1H, H-5), 4.56 (dd, J = 10.8, 1.4 Hz, 1H, H-6), 5.49 (dd, J = 9.7, 2.3 Hz, 1H, H-4), 5.83 (d, J = 2.2 Hz, 1H, H-3), 7.40 (m, 1H, Ph-H4'), 7.46 (dd, J = 8.4, 6.9 Hz, 2H, Ph-H-3', Ph-H-5'), 7.71 (d, J = 7.1 Hz, 2H, Ph-H-2', Ph-H-6'), 8.28 (s, 1H, triazole-CH); 13C NMR (75 MHz, D2O): δ 21.63 (NHCOCH3), 48.70 (C-5), 59.96 (C-4), 63.05 (C-9), 68.03 (C-7), 69.69 (C-8), 75.31 (C-6), 101.75 (C-3), 120.41 (Ph), 125.61 (Ph), 128.77 (triazole-C-5), 129.10 (Ph), 129.28 (Ph q carbon), 147.74 (triazole-C-4), 150.48 (C-2), 168.75 (COONa), 173.58 (NHCOCH3). LRMS [C19H23N4NaO7] (m/z): (+ve ion mode) 463.1 [M+Na]+; HRMS (API) (m/z): [M+1]+ calcd for C19H22N4NaO7 [M+H]+ 441.138070; found, 441.140189.
Sodium 2,6-anhydro-3,4,5-trideoxy-5-isobutyramido-4-(4-methoxymethyl-[1,2,3]triazol-1-yl)-D-glycero-D-galacto-non-2-enonate (9).

Yield = 92%. $^1$H NMR (300 MHz, D$_2$O): $\delta$ 0.98 (d, $J = 7.0$ Hz, 3H, isobut-CH$_3$), 1.03 (d, $J = 6.9$ Hz, 3H, isobut-CH$_3$), 2.46 (m, 1H, isobut-CH), 3.39 (s, 3H, OCH$_3$), 3.65–3.76 (m, 2H, H-9, H-7), 3.94 (ddd, $J = 9.3$, 6.3, 2.6 Hz, 1H, H-8), 4.49 (m, 1H, H-5), 4.60–4.65 (m, 3H, H-6, OCH$_3$), 5.61 (dd, $J = 9.7$, 2.3 Hz, 1H, H-4), 5.87 (d, $J = 2.2$ Hz, 1H, H-3). 8.18 (s, 1H, triazole-CH); $^{13}$C NMR (75 MHz, D$_2$O): $\delta$ 18.43 (isobut-CH$_3$), 18.64 (isobut-CH$_3$), 35.10 (isobut-CH), 48.19 (C-5), 57.24 (OCH$_3$), 59.86 (C-4), 63.07 (C-9), 64.24 (OCH$_3$), 68.13 (C-7), 69.82 (C-8), 75.43 (C-6), 102.02 (C-3), 123.65 (triazole-C-5), 144.07 (triazole-C-4), 150.30 (C-2), 168.81 (COONa), 180.66 (isobut-CO). LRMS [C$_{17}$H$_{25}$N$_4$NaO$_8$] ($m/z$): (+ve ion mode) 459.0 [M+Na]$^+$; HRMS (API) ($m/z$): [M+Na]$^+$ calcd for C$_{17}$H$_{25}$N$_4$NaO$_8$ [M+Na]$^+$ 459.1462; found, 459.1458.

Sodium 2,6-anhydro-3,4,5-trideoxy-5-isobutyramido-4-(4-phenyl-[1,2,3]triazol-1-yl)-D-glycero-D-galacto-non-2-enonate (10).

Yield = 89%. $^1$H NMR (300 MHz, D$_2$O): $\delta$ 0.94 (d, $J = 6.9$ Hz, 3H, isobut-CH$_3$), 0.99 (d, $J = 6.9$ Hz, 3H, isobut-CH$_3$), 2.43 (m, 1H, isobut-CH), 3.60–3.76 (m, 2H, H-9, H-7), 3.93 (ddd, $J = 12.0$, 2.7 Hz, 1H, H-9´), 4.04 (ddd, $J = 9.2$, 6.3, 2.6 Hz, 1H, H-8), 4.51 (m, 1H, H-5), 4.62 (d, $J = 11.0$ Hz, 1H, H-6), 5.58 (dd, $J = 9.7$, 2.3 Hz, 1H, H-4), 5.88 (d, $J = 2.2$ Hz, 1H, H-3), 7.42–7.54 (m, 3H, Ph-H-3´, Ph-H-4´, Ph-H-5´), 7.78 (d, $J = 7.1$ Hz, 2H, Ph-H-2´, Ph-H-6´), 8.36 (s, 1H, triazole-CH); $^{13}$C NMR (75 MHz, D$_2$O): $\delta$ 18.38 (isobut-CH$_3$),
18.65 (isobut-CH₃), 35.10 (isobut-CH), 48.23 (C-5), 59.91 (C-4), 63.07 (C-9), 68.15 (C-7), 69.76 (C-8), 75.41 (C-6), 101.96 (C-3), 120.66 (Ph), 125.67 (Ph), 128.81 (triazole-C-5), 129.16 (Ph), 129.36 (Ph q carbon), 147.71 (triazole-C-4), 150.32 (C-2), 168.80 (COONa), 180.7 (isobut-CO), LRMS [C₂₁H₂₅N₄NaO₇] (m/z): (+ve ion mode) 491.2 [M+Na]⁺; HRMS (API) (m/z): [M+Na]⁺ calc for C₂₁H₂₅N₄NaO₇ [M+Na]⁺ 491.1513; found, 491.1515.

hPIV-3 HN inhibitors: Compounds 2, 3, 5-10 were each provided as a lyophilized powder and then solubilized in sterile water to generate a 10 mM stock solution. Solutions were sonicated for 15 min to allow complete dissolution and then filter-sterilized. The stock solution was stored in a glass vial at -20 ºC and freshly diluted in appropriate buffer before use. For STD NMR experiments, stock solutions were prepared in D₂O at 100 mM. Solutions were processed and stored as described above.

Recombinant HN expression and purification: The HN protein was expressed using the Bac-to-Bac® baculovirus expression system (Invitrogen, Carlsbad, CA) based on a substantially modified literature procedure¹. Thus, the nucleotide sequence for a honeybee melittin signal peptide (HBM) was added downstream to the sequence encoding for the HN ectodomain (amino acids 125 to 572). This sequence (HBM+HN) was codon optimised for expression in Spodoptera frugiperda cells (Sf9) and ordered directly through the DNA2.0 gene synthesis service (DNA2.0, Menlo Park, CA) as a gene named HBM-HNhPIV-3opt. HBM-HNhPIV-3opt was amplified by PCR and ligated into a pFastBac®CT-TOPO® vector that provides an additional C-terminal 6-histidine tag (His-Tag) for purification and detection purposes.

The generation and amplification of recombinant baculovirus containing HBM-HNhPIV-3opt were performed according to the manufacturer's instructions. Sf9 cells (Invitrogen), cultured in Insect-XPRESS protein free insect cell medium (Lonza), were infected with high MOI of HBM-HNhPIV-3opt baculovirus. Four days post-infection the supernatant, containing recombinant HN, was collected to yield the highest protein expression. The supernatant was clarified by centrifugation (3000 RCF for 15 min) to remove cell debris and then purified on a HisTrap excel 5 mL column (GE Healthcare life sciences, Buckinghamshire, England) following the manufacturer’s protocol. Recombinant HN was eluted with 500 mM imidazole
solution and collected fractions were assessed by a neuraminidase activity (NA) assay (see below). The most active fractions were pooled and concentrated with a 10 kDa Amicon Ultra filter unit (Millipore) to a final volume of 800 µL. An additional purification step was performed that employed fast protein liquid chromatography (Amersham Biosciences) over a Superdex 75 gel filtration column (GE Healthcare) at 4 ºC and 1 mL fractions were collected with a Frac-920. Protein-containing fractions, as determined by monitoring fraction collection at 280 nm, were assessed in a NA assay as well as subjected to SDS-PAGE. Purified and concentrated recombinant HN protein was stored at 4 ºC.

**Haemagglutination inhibition (HI) assay:** The HN inhibitors were assessed in duplicate in a U-bottom 96 well plate assay. Compounds were diluted in PBS as a 4X solution for each concentration tested (25 µL per well, 1X final). Each dilution was mixed with 4 haemagglutination units (HAU) of hPIV-3 (25 µL per well, 1 HAU final) and incubated for 20 min at room temperature. The plate was transferred on ice and an equivalent volume (50 µL) of ice-cold 0.75% guinea pig red blood cells (gp-RBC) or 1% human red blood cells (h-RBC) was added to each well. The plate was then incubated for 1 h 30 min at 4 ºC before reading the extent of haemagglutination. The HI IC$_{50}$ was considered as the concentration of inhibitor that reduced the haemagglutinin activity (agglutination) by 50% compared to those of a non-treated virus suspension.

**Neuraminidase inhibition (NI) assay:** Purified hPIV-3, inhibitors and MUN were prepared and diluted in NA Reaction Buffer (NaOAc, 50 mM, CaCl$_2$, 5 mM, pH 4.6). NA, employing different hPIV-3 dilutions, were initially measured to determine the lowest virus concentration to be used in the assays. The NA assays were performed with enough purified virus to obtain a maximal fluorescence signal at least 5 times higher than the background for the experiment. Neuraminidase inhibition (NI) assays were done in triplicate. For each concentration tested, 2 µL of purified hPIV-3 and 4 µL of 2.5X inhibitor solution (1X final) was added to each well. The plate was kept at room temperature for 20 min before 4 µL of 5 mM MUN (2 mM final) was added to each well and then the plate incubated at 37 ºC for 30 min with agitation (1000 rpm). The enzymatic reaction was stopped by the addition of 190 µL of glycine buffer (glycine, 0.25 M, pH 10.4) to
each well. A negative control was included by the addition of MUN to virus and then the enzymatic reaction stopped at \( t = 0 \). Relative fluorescence (RF) was measured with a Victor 3 multilabel reader (PerkinElmer, Waltham, MA). Data were processed by background subtraction (negative control RF) and then analysed with GraphPadPrism 4 (GraphPad Software Inc., La Jolla, CA) to calculate IC\(_{50}\) values (nonlinear regression (curve fit), Dose-response - inhibition, 3 parameter logistic). The concentration of inhibitor that reduced neuraminidase activity (relative fluorescence) by 50% compared to those of a non-treated virus suspension was considered to be the NI IC\(_{50}\) value. \( K_i \) values of inhibitors 6 and 10 were determined by enzyme kinetic experiments with whole hPIV-3 virus based on previously published procedures\(^2\). Thus, neuraminidase activity was measured every 5 min over a 20 min period, at five substrate concentrations [S]: 2, 4, 8, 10 and 16 mM, and four inhibitor concentrations [I]: 0, 0.5, 2.5 and 5 \( \mu \)M for 10 or 0, 10, 20 and 60 \( \mu \)M for 6. All assays were performed in triplicate and the final data were fitted to the Michaelis–Menten equation for competitive inhibition using GraphPadPrism 4 (GraphPad Software Inc., La Jolla, CA) to determine the Michaelis–Menten constant (\( K_m \)), using data from the [I] = 0 and variable [S] experiments, and \( K_i \) values.

**SUPPLEMENTARY REFERENCES**


Appendix 3

Supplementary material to

Ren et al., 2014

This appendix contains supplementary material related to the paper:

### Supplementary Table S1 CYP inhibition assay for suramin

<table>
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<tr>
<th>Conc (µM)</th>
<th>1A2 Inhibition</th>
<th>2C9 IC₅₀ (µM)</th>
<th>2C19 Inhibition</th>
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<td>15% 56.9</td>
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Supplementary Table S2 hERG channel inhibition by suramin

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<td>Suramin</td>
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### Supplementary Table S3  Individual and mean pharmacokinetic parameters of surmanin following single intravenous bolus administration of suramin at 4.37mg/kg to male cynomolgus monkeys

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<th>Animal ID</th>
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<th>C0 (ng/mL)</th>
<th>T1/2 (h)</th>
<th>Vdss (L/kg)</th>
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# The linear regression coefficient of the concentration values on the terminal phase is less than 0.9. t1/2 might not be accurately estimated.
**Supplementary Table S4** Individual and mean plasma concentration of surmanin following single intravenous bolus administration of suramin at 4.37mg/kg to male cynomolgus monkeys.

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Note: BQL- Below the lower limit of quantitation (LLOQ).
Viral load ($\log_{10} \text{TCID}_{50}/\text{mL}$) = -0.31$ \times$ Ct + 12.50

$R^2 = 0.999$
Correlation between EV71 genome PCR CT and viral load. PCR standard curve was run for each viral quantification test. Dilute virus solution with defined titer (TCID 50/mL) serially by 10 times, extract viral RNA and apply to real time RT-PCR spontaneously with testing samples. Correlate the PCR CT with viral load to get the standard curve equation. Substitute PCR CT of testing sample into standard curve equation, so that the viral load in testing sample can be converted from PCR CT to TCID 50/mL. Data represents the means ± SEM of results of duplicated experiment.
Supplementary Figure S2 Antiviral profile of suramin. (a) Suramin reduces CVA16 infectivity. Test was replicated, and the data represents the means +/- SEM of results. (b) Poliovirus-1 is not inhibited by suramin. Data represents the result of single test.
**Supplementary Figure S3** Individual and mean plasma concentrations versus time profile of suramin following intravenous bolus administration of suramin at 4.37 mg/kg to male cynomolgus monkeys. Three monkeys were included, and were labeled as P101, P102 and P103 respectively.