Screening of a large collection of compounds for anti-human parainfluenza virus type 2 activity and evaluation of hit compounds

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Abstract

Human parainfluenza virus type-2 (HPIV-2) is a highly contagious respiratory pathogen that can cause severe respiratory disease known as laryngotracheobronchitis or croup-like disease in children. No specific vaccine or an antiviral drug is currently approved for treatment of HPIV-2 infections. In this project, a library of 14400 diverse compounds had been screened for anti-HPIV-2 activities in cultures of African green monkey kidney cells. All compounds that inhibited the virus induced syncytium-forming activity in these cells were considered as hit compounds. Three hit compounds showed moderate anti-HPIV-2 activity characterized by the IC$_{50}$ values of 20 µM and selectivity indices of approximately 5. This suggests that the antiviral activity of these compounds was due to targeting activities of cellular rather than viral components. Another hit compound, referred to as compound 5, showed anti-HPIV-2 activity that was manifested as a reduction of area of the virus-induced plaques in cells at not cytotoxic concentrations. Interestingly, this compound did not inhibit initial infection nor the virus production in infected cells as revealed by the time-of-addition assay. Moreover, it showed no direct the virus-inactivating (virucidal activity) against HPIV-2 particles. However, relatively short pre-treatment (4 hours) of the cells with compound 5 prior to the virus infection was sufficient for its plaque size-reducing activity suggesting that anti-HPIV-2 activity of compound 5 was due to targeting activities of cellular rather than viral components. Further studies are needed to elucidate the anti-HPIV-2 mechanism of activity of hit compounds identified in the present study.
Popular scientific summary

An effort to find the drug against a ‘croup disease’

Every person experience a respiratory disease at least once a year. Children and elderly use to suffer from respiratory infections more frequently and the disease is often more severe and sometimes long-lasting. Many of acute respiratory diseases are caused by viruses. The disease usually starts in the nasal cavity where the virus infects cells lining the airways, and our body responds to viral infection by triggering inflammation including production of an excess of nasal mucus that give us a breathing discomfort which facilitates the spread of the virus into environment as a consequence of frequent sneezing. The viruses expelled into environment with aerosol droplets of nasal mucus can easily infect another person. From the nasal cavity, the viral infection can also spread to the lower respiratory tract and lungs causing a more severe respiratory disease.

Some respiratory pathogens such as human parainfluenza virus type-2 (HPIV-2) can infect the cells that line the epithelium of our voice organ the larynx. The inflammation that follows the infection causes swelling of the airways in the larynx. This not only causes the breathing difficulties but the voice perturbations as well. The infected person (usually a child) have a rough, harsh or grating voice and this respiratory disease is known as a croup disease. This is a communicable disease which spreads directly person to person via sneezing and touching. This disease can be severe or even deadly in children, and an efficient vaccine or antiviral treatment are lacking. Therefore, the aim of this project was to discover a new drug for treatment of this severe disease. To increase chances of finding of such drug, in this project a large collection of 14400 random chemical compounds were tested for their capabilities to inhibit infection of cultured cells with HPIV-2. This virus destroys the infected cells by fusing their membranes so the infected cells are very large and possess numerous nuclei inside. To test such a big number of compounds a technique called the high throughput screening was used. Four out of 14400 compounds moderately protected cells from being destroyed by the virus, and these compounds can be considered as candidate anti-HPIV-2 compounds. These compounds were found to affect cultured cells so these cells are less susceptible to the virus.

One of these compounds, called hit compound 5, significantly reduced the size of virus-induced fused cells. Compound 5 did not directly destroy the virus particles but instead it affected cultured cells so they were more resistant to the virus infection and its spread to non-infected cells. All anti-HPIV-2 hit compounds identified in this project require further studies to explain the mechanism of their antiviral activity and to verify their antiviral activity in models of human respiratory infections.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CC_{50}</td>
<td>50% cytotoxic concentration</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EMEM-A</td>
<td>Eagle’s minimum essential medium (supplemented with PEST, HI-FCS and L-glutamine)</td>
</tr>
<tr>
<td>GMK AH1 cells</td>
<td>African green monkey kidney’s cells</td>
</tr>
<tr>
<td>HEP-2</td>
<td>Human epidermoid carcinoma cells</td>
</tr>
<tr>
<td>HI-FCS</td>
<td>Heat inactivated fetal calf serum</td>
</tr>
<tr>
<td>HPIV-2</td>
<td>Human parainfluenza virus type-2</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screening</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>MC</td>
<td>Methyl cellulose</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>PEST</td>
<td>Penicillin and streptomycin</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>RER</td>
<td>Rough-endoplasmic reticulum</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>ss(-)RNA</td>
<td>Single stranded negative sense strand of ribonucleic acid</td>
</tr>
<tr>
<td>ss(+)RNA</td>
<td>Single stranded positive sense strand of ribonucleic acid</td>
</tr>
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1. Introduction

1.1 Respiratory infection and HPIV

Respiratory infections in humans are frequently referred to as community-acquired infections due to the fact that viral and bacterial pathogens which cause these infections are highly contagious especially when someone is present in various community events such as collective transport, public gatherings, etc. These pathogens can spread via airborne droplets of respiratory fluids or through the contact with respiratory secretions located on solid surfaces. People get multiple respiratory infections in their life which can be severe especially in children and elderly. The viruses that most frequently cause acute respiratory infection include influenza virus, parainfluenza viruses, respiratory syncytial virus, certain coronaviruses, adenoviruses and others. Human parainfluenza viruses (HPIVs) are classified into the family Paramyxoviridae. HPIVs presently consist five serotypes of which HPIV-1 and HPIV-3 form a Respirovirus genus while HPIV-2, HPIV-4, and HPIV-5 belong to Rubulavirus genus.

HPIVs usually spread from an infected person to other individuals through the aerosol droplets generated by coughing and sneezing or by touching objects or surfaces covered with respiratory fluids from infected person (Schomacker et al., 2012). HPIVs can be infectious in airborne droplets for over an hour and on solid surfaces for a few hours. These viruses are most contagious during the early stage of illness when respiratory fluids contain no specific antibodies or inflammation mediators. HPIVs are highly infectious and target the cells lining the airways frequently resulting in serious diseases of upper and lower respiratory tract. HPIVs frequently cause an acute infection of the lower respiratory tract in younger children from 6 months to 5 years of age (Reed et al., 1997). Some recent comparative studies of acute infections of lower respiratory tract caused by HPIVs and respiratory syncytial virus (RSV), showed a higher death rate values in children infected with HPIVs (8.9%) than with RSV (1.3%) (Pecchini et al., 2015).

1.2 HPIV-2

HPIV-2 was first isolated from child with a croup-like disease and therefore this virus is frequently referred to as a croup virus. The croup disease is in fact an acute inflammation and swelling of the larynx, trachea and bronchi which may result in serious respiratory discomfort due to partial obstruction of airways clinically known as laryngotracheobronchitis (Schomacker et al., 2012). As with other HPIVs, this disease is mostly found in young children and immunocompromised persons. The major symptoms of the disease in children are difficulties with breathing, characteristic “barking” a cough, grating breathing known as stridor, and a weak hoarse voice due to inflammation of the larynx (Rajapaksa and Starr, 2010).

HPIV-2 particles are pleomorphic enveloped virions (Howe et al., 1967) which contain a lipid envelope covering the viral genetic material represented by a single, negative sense, RNA strand (Fig. 1). The outermost components of the viral particle are the virus-specific glycoprotein spikes that are anchored in the lipid envelope. These spikes consist either the hemagglutinin-neuraminidase (HN) or the fusion (F) protein which play an important role in the virus attachment to susceptible cells (HN protein) and fusion between the viral lipid envelope and the cell plasma membrane (F protein) respectively.
The primary function of HN protein (the hemagglutinin component) is to mediate adsorption of the virus particle to sialic acid containing glycoproteins or glycolipids on the cell surface. Sialic acid, also known as neuraminic acid, is a terminal sugar residue on glycan chains of these cell surface components. The neuraminidase component of the viral HN protein is an enzyme that mediates cleavage of sialic acid from the cell surface receptor molecules (glycoproteins or glycolipids). Binding of neuraminidase-defective virus to cellular receptors results in prolonged trapping of viral particle on the surface of the cells instead of their infection (Palese et al., 1974; Yuan et al., 2005).

![Schematic structure of HPIV-2 particle.](image)

**Fig. 1. Schematic structure of HPIV-2 particle.** The helical viral RNA encapsidated with nucleoprotein (nucleocapsid) is covered by the lipid envelope (in blue). The viral glycoproteins (HN, F, SH) which protrude from the virus surface are embedded in the virus envelope. The viral matrix protein is located just beneath the viral envelope to shape the virus particle and to crosslink the viral glycoprotein with nucleocapsid protein.

Neuraminidase activity also prevents self-aggregation of viral particles during the budding stage of the virus by cleaving sialic acid residues of the virus own glycoproteins (Palese et al., 1974). The F protein promotes fusion between lipids of the virus envelope and the cell plasma membrane thus resulting in the introduction of viral genetic material into cytoplasm. At the late stages of viral infection of cells, the F protein is also expressed on the plasma membrane of the infected cells. The plasma membrane can fuse with adjacent plasma membrane of non-infected cells to form syncytia (multinucleated cells).

The F protein is synthesized as precursor (F0), that is cleaved to its active form (F1, F2) by proteolytic enzymes of the host. Thus, proteolytic activation is a critical event for HPIVs pathogenesis and tropism, because cells lacking this activity are not able to promote the virus replication.
Embedded in the virus envelope is also a small hydrophobic (SH) protein with ion-channel activities important in the virus budding from infected cells. The matrix protein (M) represents a scaffold of the viral particle and is located just beneath the virus envelope. The protein is hydrophobic with some positively charged residues and acts as mediator of interaction between the glycoproteins and the nucleocapsid. Due to these activities, the M protein seems to be important during assembly and budding of the virus particles from cells.

The HPIV nucleocapsid comprise the viral genome, i.e., non-segmented, single-stranded RNA of negative sense polarity which is covered with the nucleoprotein (N). Paramyxoviruses cover their genomic RNA with the N protein to protect it against digestion with cellular ribonucleases, to prevent induction of interferon system of the cell, and to help in assembly of viral particles. The amino-terminal ¼ portion of the protein is involved in interaction with viral RNA and it is also involved in binding to other copies of the N-protein (self-assembly), an activity that is crucial in assembly of viral nucleocapsid chain (Fookes et al., 1993; Myers et al., 1997, 1999; Nishio et al., 1999; Schoehn et al., 2004; Buchholz et al., 1993).

The C-terminal portion of the N protein is believed to attract the viral-phosphoprotein polymerase complex (Kingston et al., 2004; Buchholz et al., 1994) to initiate the virus replication. The viral phosphoprotein (P protein) contains numerous serine and threonine residues some of which are phosphorylated, a modification that is crucial for the biological activity of this protein (De et al., 1995). As mentioned above, the P protein interacts with the N protein to help in encapsidation of viral RNA (Buchholz et al., 1993), and to link the viral polymerase with nucleocapsid (Buchholz et al., 1994). Because viral RNA is encapsidated with the N protein, the major function of the P protein is to help the viral polymerase in movement along the encapsidated viral RNA template (Blanchard et al., 2004; Bourhis et al., 2006).

The viral large (L) protein is a principal component of viral RNA-dependent RNA polymerase that given its catalytic activity occurs in infected cells in much fewer copies than other viral proteins. The L protein forms an active enzyme complex with the P protein (Hamaguchi et al., 1983) and both proteins are frequently found colocalized on the nucleocapsid (Portner et al., 1988). The L protein possesses all enzymatic activities required for synthesis of functional viral mRNAs which includes 5´-end capping/methylation and polyadenylation at the 3´end (Ogino et al., 2005). The viral V protein consists of an N-terminal region that is identical with the viral P protein and the C-terminal region known as cysteine-rich domain that is specific for the V-protein. The protein plays a role in pathogenesis of HPIV infection as well as in evasion of the antiviral response in infected cells (Andrejeva et al., 2004).

1.3 Stages of HPIV-2 replication in susceptible cells as targets for antiviral intervention

The first step in the life cycle of HPIV is attachment of the viral particle to susceptible cells (Fig. 2; step 1). HPIV binds to sialic acid component of cellular glycoproteins or glycolipids located at the cell plasma membrane. The binding is performed by the viral attachment protein known as HN. This protein contains neuraminidase activity to help to release virus particles that were trapped by too strong binding to distant cellular components (e.g. mucins) or were self-aggregated by binding to sialic acid on their own glycoproteins (Palese et al., 1974).
This event of viral life cycle appeared to be highly sensitive for treatment of influenza virus (the sialic acid binding virus) infection as the drugs approved for this virus are inhibitors of viral neuraminidase activity (Von Itzstein et al., 1993).

In the next step of HPIV life cycle, the cell-attached virus enters the cell by fusion between viral envelope and the cell plasma membrane. The viral F protein is a fusion device that inserts a so called fusion peptide into cellular membrane which is followed by conformational rearrangement of the protein to bring the viral and cellular membrane in close proximity (Fig. 2; step 2) and to fuse them (Fig. 2; step 3). This fusion event was extensively exploited for antiviral intervention against another paramyxovirus, the respiratory syncytial virus (RSV) (Lundin et al., 2010), and the best candidate drug, the fusion inhibitor GS-5806 protected human volunteers against experimental RSV infection (De Vincenzo et al., 2014).

Following fusion of viral and cellular membranes, the viral nucleocapsid is inserted into the cells. HPIV replication occurs in the cytoplasm of the host cell. The viral nucleocapsid is subjected to primary transcription by viral polymerase where the viral RNA serves as a template for production of different species of mRNA which are then translated to viral proteins (Fig. 2; step 4a) (Fig. 3). When the accumulation of viral proteins is high, the viral nucleocapsid undergoes replication into full length antigenome (RNA+) that functions as a template for production of a new copy of viral genome (Fig. 2; step 4b) which in turn is subjected to secondary transcription to produce more viral components (Fig. 2; steps 5, 6, and 7) (Fig. 3) required for assembly of progeny virions.

Fig. 2. HPIV-2 life cycle. The virus particle attaches to sialic acid containing glycoproteins or glycolipids (1). Following fusion between the lipids of viral envelope and cell plasma membrane (2 and 3), the viral nucleic acid is released into cytoplasm where it undergoes transcription into mRNAs (4a) for production of viral proteins (5 and 6). When the level of protein production is sufficient, the viral RNA undergoes replication in to antigenomic RNA (+) and genomic viral RNA (-) (4b). Both viral proteins (7) and viral RNA (-) are transported to the cell membrane where assembly of viral particles takes place (8). The progeny viral particles are formed during their budding (9) from the cell plasma membrane. Finally, the newly formed viral particles are released from the cell (10).
Transcription and replication of paramyxovirus genome including the enzymatic activities of viral L polymerase protein required for capping, methylation, and polyadenylation of viral RNA have been extensively exploited as targets for antiviral intervention (Mao et al., 2008) however none of the tested compounds has so far been approved for antiviral treatment. The virus assembly takes place at specific regions of the cell plasma membrane where the inserted viral HN, F, and SH proteins attract the viral M protein to the cytoplasmic side of the membrane which in turn serves as a template for specific incorporation of the viral nucleocapsid (Fig. 2; step 8) The assembled viral components are released from cells by budding from the cell plasma membrane (Fig. 2; step 9). Again, the neuraminidase activity of the viral HN protein is required to liberate viral particles from their binding to sialic acid containing components at the cell plasma membrane (Fig. 2; step 10).

In humans, HPIV-2 targets and replicates in the ciliated epithelial cells (Schaap-Nutt et al., 2010) that line the respiratory tract and transport the mucus towards the nasal cavity. Infection of ciliated cells by the virus causes their dysfunction that slows down the mucus transport. This condition together with inflammation of larynx and trachea may lead to partial or complete obstruction of airways (Schomacker et al., 2012). Several candidate HPIV vaccines (Joanes et al., 2009) and candidate anti-HPIV drugs (Watanabe et al., 2009) had been evaluated but none of them have been approved for prevention and/or treatment of the infections caused by HPIVs.

Fig. 3. Strategy for replication of HPIV-2 genome. For explanations see the text.
Thus, in spite of the fact that acute viral respiratory infections are common in humans, there is no treatment (excluding influenza virus) against these pathogens. Most of the approved antiviral drugs are specific against dangerous non-respiratory human viruses such as human immunodeficiency virus and hepatitis C virus (De Clercq, 2013).

The aim of the study is to identify novel anti-HPIV-2 candidate antiviral compounds, and to evaluate their antiviral potency. Therefore, a cell culture based method (Lundin et al., 2013) was used for screening of a large collection of diverse compounds for anti-HPIV-2 activity. This method has been successfully used for identification of novel drugs P13 and C15 (fusion inhibitors) against RSV (Lundin et al., 2010), and a drug K22 against coronaviruses (Lundin et al., 2014). The screening assay was performed in GMK AH1 cells where infection with HPIV-2 causes a characteristic cytopathic effect, i.e. formation of syncytia that is clearly detectable when observed under a microscope. Any compound that was capable of protecting the cells against the HPIV-2 induced syncytia of cells was regarded as a “hit” and subjected to further evaluation of antiviral potency and mechanism of antiviral activity. In the present study, four novel anti-HPIV-2 compounds were identified including compound 5 that reduced the cell-to-cell spread of HPIV-2 infection. These compounds will be subjected to further evaluation of their antiviral mode-of-activity.
2. Materials and methods

2.1 Cell culture media and other reagents

Eagle’s minimum essential medium (EMEM) was purchased from Gibco. Fetal calf serum (FCS), purchased from Sigma, was heat-inactivated by incubation for 30 min at 56°C in a water bath (HI-FCS). Penicillin (10 mg/ml) and streptomycin (6 mg/ml) (PEST) were dissolved in Hank’s balanced salt solution, and the stock was sterilized by filtration. The L-glutamine was dissolved in deionized water (29.2 mg/ml) and the stock was sterilized by filtration. Primaton RL substance was purchased from Kraft Inc., Norwich, CT, USA. Methyl cellulose (Methocel® MC, Sigma, 64625) stock was prepared by dissolving 1.5 g of the powder in 100 ml of Hank’s balanced salt solution and sterilized by autoclaving at 120°C for 20 min. Methyl cellulose overlay medium (MC) was prepared by mixing equal volumes of methyl cellulose stock solution with EMEM supplemented with 1.5% PEST, 1% L-glutamine, and 1% HI-FCS. Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, and 0.5 mM MgCl₂), was prepared by dissolving each component in deionized water. Glutaraldehyde was purchased from Sigma (G7651). Dimethyl sulfoxide (DMSO) was obtained from Sigma (D4540). Crystal violet stock solution was prepared by dissolving 1 g of crystal violet in 87 ml of 70% ethanol supplemented with 8.7 ml of formaldehyde (37% aqueous solution, F1635, Sigma) and 4.3 ml of glacial acetic acid. CellTiter 96® Aqueous One Solution reagent (G3580) for cytotoxicity measurement was purchased from Promega (Madison, WI, USA).

2.2 Cell culture

African green monkey kidney (GMK AH1) (Gunalp, 1965) cells were propagated in EMEM supplemented with 2% FCS, 0.05% Primaton RL substance, and 1% PEST. The cells were grown at 37°C in humified atmosphere comprising 5% CO₂.

2.3 Virus propagation and isolation of HPIV-2 from clinical specimens

Human parainfluenza virus type 2 (strain, KS/70-C517) obtained from Karolinska Hospital (Stockholm) was used throughout the experiments. The virus stock was prepared as follows. GMK AH1 cells, seeded in 162 cm² bottles the day prior to the experiment, were rinsed once with 12 ml of the EMEM supplemented with 1% PEST, and 25 ml of the same medium was added. The virus stock (0.5 ml) was quickly thawed in the water bath at 37°C and added to the cells. The cells were gently rotated every 30 min during the 3 h period of incubation at 37°C to facilitate adsorption of the virus. The medium was removed and 25 ml of the fresh medium comprising EMEM supplemented with 1% PEST, 1% L-glutamine, and 2% HI-FCS was added. Then, the bottles with cultured cells were placed at 37°C in the CO₂ incubator for up to 3 days until a characteristic cytopathic effect (CPE) (massive syncytia) was evident based on microscopic examination. The infected cells were shaken vigorously, and the infectious culture medium was harvested and centrifuged at 1000xg for 5 min. The supernatant medium was then collected, aliquoted in 0.5 ml volumes, and stored as virus stock at -80°C.
Isolation of HPIV-2 was performed using clinical specimens of nasopharyngeal fluid collected from eight different patients suffering from respiratory disease. These specimens were diagnosed at the Department of Clinical Virology, Sahlgrenska Hospital/University of Gothenburg, as being positive for HPIV-2 by Taqman PCR. The clinical specimens were transferred to separate tubes and supplemented with 50 µl of PEST and 50 µl of nystatin stock (10000 Units/ml; Sigma). The medium was removed from the GMK AH1 cells, that were seeded the day prior to the isolation in 25 cm² bottles, and the clinical specimens along with 5 ml of fresh EMEM-A medium were added into the bottles. The cells were incubated for 24 h at 37°C. After 24 h the cells were rinsed with 5 ml of the same medium and 5 ml of the fresh medium was added. The cells were observed for CPE for 7 days on the daily basis by using the light microscope.

2.4 Compound collection (library) for antiviral screening

The HitFinder diversity library of 14400 compounds in 384 plate format was obtained from Maybridge (UK). All compounds were supplied as a film containing 0.25 µmole of the compound. The compounds were solubilized in 12.5 µl of DMSO and split into three identical copies by transferring 4.1 µl of solubilized compound to two other empty plastic 384 well plates (catalog #3657; Corning-Costar). Then, 36 µl of sterile redistilled water was added, the plates were briefly centrifuged, covered with the DMSO-resistant aluminum film (catalog # CLS-6569, Thermowell™ Sealing Tape, Costar-Corning) and stored at -20°C. The final concentration of compounds was 2 mM.

2.5 Virus titration for antiviral screening

The suspension of GMK AH1 cells was added at 50 µl volumes into each well of a sterile 384-well plate (Corning, CLS-3701) by using a 16-channel pipette, and the plates were centrifuged at 100xg for 30 secs to remove any air bubbles in the wells. Next, the plates were incubated in the CO₂ incubator (humidified atmosphere comprising 5% CO₂) at 37°C for 24 h. The next day, when the cell monolayers were ~80% confluent, the growth medium supernatant was removed from cells by inverting the plate over a waste container, and 50 µl of an assay medium comprising EMEM, 1% HI-FCS, 1% L-glutamine, and 1.5% PEST (EMEM-A) was added. The medium was removed from cells and 25 µl of fresh EMEM-A was added. Subsequently, 1 µl of 10% solution of DMSO solution (the solvent for library compounds) was added using a 16-channel micropipette. The plates with cells were then shaken for 1 min at 750 cycles (Heidolph Titramax 100 shaker) and placed in the CO₂ incubator. Serial 10-fold dilution (from 10⁻¹ to 10⁻⁹) of the virus stock were performed in an empty 24 well plates and 25 µl of each dilution was transferred to cells growing in 32 separate wells in 384 well plates. The plates were centrifuged at 100xg for 30 secs, then shaken for 1 min at 750 cycles and placed for 5-6 days in the CO₂ incubator. Finally, the cells were inspected under the microscope for the presence of the virus-induced cytopathic effect (CPE), and the virus tissue culture infectious dose titer was calculated (Reed and Muench.1938).

2.6 Antiviral screening

For screening of the library of compounds, a previously described method (Lundin et al, 2013) was adopted. At the day of the experiment, the plates with library compounds were placed at 37°C for 15 min to melt the compound solution, then centrifuged at 100xg for 30 secs, and shaken for 1 min at 750 cycles to re-suspend any insoluble (pelleted) compounds.
Next, the aluminum foil cover was removed from library plates and 1 µl of each compound was transferred to corresponding wells with cells by using a 16-channel micropipette, and the plates were shaken to redistribute the compound. Then, 25 µl of the virus stock suspension comprising 330 TCID₅₀ in EMEM-A was distributed into each well of the plate except for wells in columns 1 and 24 where 25 µl of EMEM-A was added. Again, the plates were centrifuged at 1000xg for 30 secs, shaken for 1 min at 750 cycles, and placed for 5-6 days in the CO₂ incubator. The cells in columns 1 and 24 comprised no virus and no compounds (the cell control) while the cell in column 2 and 23 received the virus only (the virus control). All wells with cells were inspected under the microscope for the presence of the virus-induced CPE, and any protection, related to the virus or the cell control, was recorded.

2.7 Validation of hit compounds

The assay was performed in 24 well plate format. The GMK AH1 cells were seeded at 1 ml per well of cell suspension adjusted to give ~80% confluency of 1-day old cultures. The compounds that were found in the screening assay as showing some anti-HPIV-2 activity were 5-fold diluted in EMEM-A to receive the final concentrations of 200 µM, 40 µM, and 0 µM. The medium was removed from the plate with the cells. Then, the cells were rinsed once with 0.5 ml of the fresh EMEM-A, and 180 µl of EMEM-A was added. Twenty microliters of diluted hit compound were added to cells to achieve the final concentration of 20 µM, 4 µM, and 0 µM (the virus control). The virus stock was diluted in EMEM-A and 25 µl of this suspension containing ~100 plaque forming units (PFU) was added. After 2 h incubation of the virus-compound mixture with the cells, 600 µl of MC overlay medium comprising the same concentration of hit compounds was added. The plates were placed in the CO₂ incubator for 6 days, and then the cells were stained with 1% crystal violet solution.

2.8 Anti-HPIV-2 activity of selected hit compounds (IC₅₀)

The assay was performed by the viral plaque number reduction assay in the 24 well plate format (Lundin et al, 2010). The GMK AH1 cells were prepared for this experiment as described in above section. At the day of the experiment, the culture media were removed from GMK AH1 cells which were then rinsed once with 0.4 ml of EMEM-A, and 180 µl of fresh EMEM-A was added. Meanwhile, serial five-fold dilutions of selected hit compounds were prepared in an empty 24 well plate by adding/transferring 40 µl of hit compound to 160 µl of EMEM-A to receive 400, 80, 16, 3.2, 0.64 and 0.0 µM. Twenty microliters of these dilutions were added in duplicates to cells comprising 180 µl of EMEM-A to achieve a final concentration of 40, 8, 1.6, 0.32, 0.064, and 0.0 µM. The last dilution comprised no compound to serve as a control. Then, 25 µl of diluted virus (~100 PFU) was added to each well and the rest of the procedure was as described in chapter 2.7.

2.9 Cytotoxicity of selected hit compounds (CC₅₀)

The effect of test compounds on the viability of cells was tested by using the tetrazolium (MTS)-based cell proliferation assay (Lundin et al, 2013). This assay measures metabolic activity of the cells based on the formation of brown formazan product. Briefly, 200 µl of GMK AH1 cell suspension was seeded into each well of 96 well plates the day prior to the experiment. Five-fold serial dilutions of hit compounds were performed in empty 96 well plate in a total volume of 200 µl of EMEM-A to achieve final concentrations of 40, 8, 1.6, 0.36, 0.064, and 0.0 µM (the control sample).
The culture medium was removed from cultures of GMK AH1 cells which were then rinsed once with 200 µl of EMEM-A, and then 100 µl of working dilutions of the hit compounds were added in duplicates to cells. Following incubation of cells in the CO₂ incubator at 37°C for 72 h, 20 µL of the Cell Titer 96® AQ reagent (Promega) was added to each well and incubated with cells for further 1-2 h. Finally, the results were recorded by using the spectrophotometry (Thermo Scientific Multi-scan FC) with measurement of developed formazan product at 492 nm against the background of 650 nm.

2.10 Plaque size reduction assay

The activity of hit compound that reduced the size of viral plaque by 50% (IC₅₀) was determined by the plaque size reduction assay (Lundin et al, 2012). The virus-induced plaques that were developed in GMK AH1 cells in the presence of specific concentrations of test compound in MC overlay medium were stained with 1% of crystal violet solution. The images of 30 plaques (15 from two replicate wells) were captured using a Leica DC 300 digital camera attached to a Leitz-Wetzlar Diavert microscope. The IM 1000 image software (Leica) was used to measure the area of each plaque.

2.11 Electron microscopy

The GMK AH1 were seeded in 24 well plate on the Melinex plastic coverslips and incubated in the CO₂ incubator for 1 day. The monolayers of cells (1 x 10⁵ cells) were rinsed once with 0.5 ml of EMEM-A, and the cells in separate wells were infected with the HPIV-2 stock virus at a multiplicity of infection (moi) of 5, 0.5 and 0.0 PFU/cells. The cells were incubated for 3 days at 37°C in the CO₂ incubator. After incubation, the cells were fixed with 0.5 ml of a warm solution of 2.5% glutaraldehyde in EMEM at 37°C for 30 min. Next, the medium was collected from each well, and the cells were further fixed for 1 h at room temperature with 1 ml of 2.5% glutaraldehyde in Sorensons 0.2 M phosphate buffer. Finally, the cells were rinsed twice with 1 ml of 0.05 M Tris-HCl buffer (pH 7.4) supplemented with 2 mM CaCl₂ and stored in 0.5 ml the same buffer until processed for electron microscopy (Widehn and Kindblom, 1990).

2.12 Cellular effects of compound 5

The effect of prior treatment of GMK AH1 cells with compound 5 was carried out in cells growing in the 24 well plate format. The medium was removed from the cells which were then rinsed once with 1 ml of the EMEM-A medium, and 1 ml of working dilutions of the test compound at 20, 10, 5, and 0.0 µM were added (in triplicate) to cells. The plates were incubated for 4 h at 37°C in the CO₂ incubator. The medium was removed, the cells were rinsed twice with 1 ml of fresh medium, and 0.5 ml of the same medium was added. Subsequently, ~200 PFU of the virus in 25 µl of EMEM-A medium was added to cells. The plates were placed in the CO₂ incubator for 2 h. The medium was then removed and overlaid with 1.5 ml of MC solution. The plates were incubated for 6 days at 37°C, whereafter the cells were stained with 1% crystal violet solution to visualize the HPIV-2 plaques.
2.13 Virucidal activity of compound 5

The HPIV-2 at 5 x 10^4 PFU in 0.5 ml of EMEM-A medium were mixed in 2-ml vials with 5 µl of EMEM-A medium containing compound 5 at 10000, 2000, or 0.0 µM. The mixtures were incubated for 15 minutes at 37°C in the water bath. Then, the residual viral infectivity was titrated by the viral plaque assay as follows. The mixture of virus and compound was subjected to serial 10-fold dilutions by adding and then transferring 30 µl of the mixture to 270 µl of the EMEM-A medium in the 96 well plate format. Subsequently, 50 µl volumes of these dilutions were added to duplicate wells with GMK AH1 cells propagated in 0.2 ml of EMEM-A in 24 well plates. The plates were placed for 3 h at 37°C in the CO2 incubator, whereafter the cells were overlaid with 0.6 ml of MC solution. After incubation for 6 days at 37°C in the CO2 incubator, the cells were stained with 1% crystal violet solution and the viral plaques counted under a microscope.

2.14 Time-of-addition assay

To investigate which step of HPIV-2 life cycle was targeted by the compound 5, this hit compound was diluted in warm EMEM-A medium to 100 µM concentration. One-day old cultures of GMK AH1 cells were rinsed once with 0.5 ml of the EMEM-A medium, and 425 µl of the same medium was added. Subsequently, 1 x 10^5 PFU of the virus in 25 µl of EMEM-A was added to cells to achieve a moi of 1 PFU/cells. The cells were incubated for 1 h at 37°C, then rinsed twice with 0.5 ml of the EMEM-A medium and 0.5 ml of the same medium was added. The compound 5 in 50 µl volumes was added at different time-points (0 h through 8 h) relative to the infection of cells with HPIV-2. The 0 h and 1 h represent the time of compound addition just prior to the virus addition to cells and just after removal of the virus from cells respectively. The cells were incubated for 3 days at 37°C in the CO2 incubator and the yield of the virus in the supernatant medium was titrated as described in the “Virucidal assay”.

2.15 Statistical analysis

Where stated, unpaired two-tailed Student’s t-test was used for statistical analysis. The Reed and Muench method (1938) was used for calculation of 50% endpoint of viral titers (TCID_{50}). Graphs were created using the Sigma-Plot software while the statistical analysis of the data was carried out using the Microsoft Excel.
3. Results

3.1 Identification of morphological features of virus-induced CPE required for antiviral screening

The aim of this research project was to search for novel compounds showing antiviral activities against the HPIV-2 virus. This task was performed by screening the HitFinder diversity collection (library) of compounds for their capability to protect the GMK AH1 cells against the virus-induced CPE. Therefore, prior to screening experiments, the features of the virus-induced CPE in cells were investigated by light and electron microscopy (See method in section 2.11). It was found that HPIV-2 induced in GMK AH1 cells syncytia of the membranes of infected cells, i.e., the formation of large cells (poly-karyocytes) containing numerous (>10) nuclei (Fig. 4A). These nuclei were preferably located at the periphery of poly-karyocytes. The virus-induced syncytia/poly-karyocytes were first visible at 2 days after infection of cells and their size gradually increased until day 6 post-infection. At the ultrastructural level, the virus caused in infected GMK AH1 cells substantial enlargement (proliferation) of the rough endoplasmic reticulum (Fig. 4B). This alteration most likely represents the virus replication center that helps the virus to produce the viral proteins and nucleic acid for assembly of progeny viral particles (virions). The assembly of progeny viral particles took place at cell plasma membrane (Fig. 4C) where the viral membrane and nucleocapsid strands were clearly seen as dark spots of perpendicularly cut strands. The released viral particle had elongated shape (Fig. 4D) with the unique pattern of nucleocapsid strands arrangement seen as dark spots or long strands at perpendicular and parallel cuts respectively.

Fig. 4. Features of cytopathic effect (CPE) induced by HPIV-2 in GMK AH1 cells. (A) The virus induced plaques in form of syncytia/poly-karyocytes. Note presence of numerous nuclei seen as white spots at the periphery of poly-karyocytes. (B) Electron microscopy image of HPIV-2 induced changes in the cytoplasm of infected GMK AH1 cells in form of dilated and proliferated rough endoplasmic reticulum denoted with a white arrow (N-nucleus; M-mitochondrion). (C) HPIV-2 particle budding from the cell plasma membrane (white arrow). The dark spots most likely represent the viral nucleocapsid. (D) The viral particles of HPIV-2. The dark spots on the left image most likely represent perpendicularly cut viral nucleocapsid which is visible as a pattern of long strings in parallelly cut virus particle on the right image. A total of 67 images were captured and the representative images are shown.
3.2 Antiviral screening; overview, adjustment of the virus dose and criteria for hit identification

Having identified features of the HPIV-2 induced CPE, the screening of the HitFinder collection was initiated as schematically illustrated in the flowchart of the screening procedure (Fig. 5). The virus dose to be used in screening was adjusted as described in section 2.5. Based on this virus titration it was decided to use in antiviral screening 330 TCID<sub>50</sub> doses per well. TCID<sub>50</sub> is a statistically calculated dose (Reed and Muench, 1938) (See method in section 2.15), and this specific TCID<sub>50</sub> virus dose was selected for screening to assure the stable and reproducible appearance of viral CPE. The results of screening assay were recorded on day 6 after virus infection by using a light microscopy (See method in section 2.6).

Fig. 5. Schematic overview; Screening and the hit validation/optimization experiments.

The wells in columns 1 and 24 comprised DMSO only and served as a control of non-infected cells (Fig. 6A) while the wells in columns 2 and 23 contained DMSO and the virus to serve as the control of the virus-induced CPE (Fig. 6B). The remaining wells in 384 plate comprised a specific compound and the virus, and any protection of cells by a screened compound was always compared to the virus control (Fig. 6B; no protection=full CPE) and the uninfected cell control (Fig. 6A; no viral CPE=full protection).
The results were accepted as valid only when all the 32 wells serving as virus controls showed extensive CPE and all 32 wells serving as cell controls exhibited no sign of infection or cytotoxicity. A substantial number of library compounds adversely affected GMK AH1 cells by causing decreased proliferation of cells (cytostatic activity) (Fig. 6C) or by being directly toxic to cells which were manifested as their disruption and shrinkage (Fig. 6D). Identification of such compounds was important since cytostatic effect of these compounds on cells may protect the cells against the viral CPE thus leading to identification of false positive hits.

Fig. 6. Morphology of GMK AH1 cells under physiological and pathological conditions found in the screening assay. (A) Normal GMK AH1 cells. (B) HPIV-2 induced the cytopathic effect in cells in form of large syncytia (white arrow). (C) Cytostatic effect of a screened compound. Note poor density of the cell monolayer and altered cell shapes. (D) Cytotoxic effect of a screened compound. Note shrinking of cells and a dark staining. Because it was difficult to capture satisfactory images of unstained cells from 384 well plate format of the screening assay, all images shown were captured from 24 well plate format comprising 6 days old monolayer cultures of cells stained with 1% crystal violet solution.

3.3 Identification of anti-HPIV-2 hit compounds

To identify novel anti-HPIV-2 candidates drug, a large library of compounds was screened for antiviral activity (See methods in sections 2.4 and 2.6). The results of the screening experiments are shown in Table 1. A total of 402 (2.8%) out of 14400 compounds were identified in initial screening as showing some protection of GMK AH1 cells against the virus-induced CPE. Because these protective activities could still be due to more or less apparent cytostatic properties, the antiviral activity of all these hits was validated in a larger, 24 well plate format.
Here, the initial hit compounds were retested for their protective effects against the viral induced plaque-forming activity (See method in section 2.7). The hit compounds were tested at concentration 20 and 4 µM, and the number of plaques developed under these conditions was compared to the number of plaques found in cells incubated in the absence of hit compounds. Based on the results, 20 compounds including those that showed good antiviral protection in the initial screening and little activity in the validation assay were selected for further detailed examination of their antiviral activities and their effect on the cell viability (Table 2).

<table>
<thead>
<tr>
<th>Protection of cells against HPIV-2 induced CPE</th>
<th>Without apparent cytostaticity</th>
<th>With cytostaticity</th>
<th>Microbial contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete protection</td>
<td>8*</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Partial protection</td>
<td>394</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>No protection</td>
<td>13917</td>
<td>8</td>
<td>25**</td>
</tr>
</tbody>
</table>

*Number of hits identified.
**Due to the fact that the screening libraries are not fully sterile and microbial contamination may occasionally occur.

Table 2. Anti-HPIV-2 activities and cytostatic properties of selected hit compounds

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>Library (plate-location)</th>
<th>Antiviral activity (IC&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Cytotoxicity (CC&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Selectivity Index (CC&lt;sub&gt;50&lt;/sub&gt;/IC&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-F16</td>
<td>20</td>
<td>&gt;100</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>4-O6</td>
<td>&gt;40</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>5-N3</td>
<td>30</td>
<td>&gt;100</td>
<td>&gt;3.3</td>
</tr>
<tr>
<td>4</td>
<td>5-O4</td>
<td>40</td>
<td>&gt;40</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>9-M10</td>
<td>40</td>
<td>&gt;40</td>
<td>&lt;0.75</td>
</tr>
<tr>
<td>6</td>
<td>13-J16</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>14-O13</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
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<td>9</td>
<td>21-C11</td>
<td>&gt;40</td>
<td>&gt;100</td>
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<td>23-F18</td>
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<td>90</td>
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<td>&gt;100</td>
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<td>32-I8</td>
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<td>&gt;100</td>
<td>&gt;5</td>
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<tr>
<td>15</td>
<td>39-H4</td>
<td>20</td>
<td>90</td>
<td>4.5</td>
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<tr>
<td>16</td>
<td>39-H8</td>
<td>&gt;40</td>
<td>80</td>
<td>&lt;2</td>
</tr>
<tr>
<td>17</td>
<td>40-C8</td>
<td>&gt;40</td>
<td>&gt;100</td>
<td>2.5</td>
</tr>
<tr>
<td>18</td>
<td>42-A3</td>
<td>&gt;40</td>
<td>&gt;100</td>
<td>2.5</td>
</tr>
<tr>
<td>19</td>
<td>42-M10</td>
<td>&gt;40</td>
<td>&gt;100</td>
<td>2.5</td>
</tr>
<tr>
<td>20</td>
<td>44-F4</td>
<td>5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Not determined
3.4 Antiviral potency of identified hit compounds

All promising hit compounds selected in section 3.3, were examined for anti-HPIV-2 activity in a dose-depended manner (0-40 µM) by the viral plaque reduction assay (See method in section 2.8) (Fig. 7). Ten out of 20 compounds exhibited statistically significant inhibition of HPIV-2 infection in GMK AH1 cells when used at a concentration of 40 µM, and hit no. 20 also substantially reduced the viral infectivity at 8 µM (Table 2, in bold). Under the experimental condition of this assay, the remaining compound showed little or no effect on protection of cells against this virus. The concentrations of active hit compounds that reduced the number of developed viral plaques by 50% (Inhibitory concentration 50%; IC₅₀) were extrapolated from the dose-response curves (Fig. 7) and their values are shown in Table 2.

![Graphs of antiviral potency](image)

**Fig. 7. Anti-HPIV-2 activities of selected hit compounds.** GMK AH1 cells growing in 24 well plate were infected with 100 plaque forming units (PFU) of the virus in the presence of specific concentration of selected hit compounds specified in Table 2. The results are presented as a percentage of a number of viral plaques (PFU) found in cells incubated with the virus and the compound as relative to control wells that received the virus only. The data shown are means of duplicate determinations. All data points showing statistically significant difference are denoted with asterisks with the following P-values: *P<0.05, **P<0.01, and ***P<0.001.
3.5 Cytotoxic activities of identified hit compounds

Because inhibitory activities of these hit compounds could be due to their more or less apparent adverse effects on cells, all these compounds were examined in the tetrazolium-based cell viability assay (See method in section 2.9). Three of those compounds (no. 10, 13, and 14) showed moderate but statistically significant cytostatic activity for GMK AH1 cells at 100 µM while compounds no. 8 and 20 appeared to be strongly cytostatic for GMK AH1 cells by reducing cell viability at ≥1.6 and ≥0.8 µM respectively (Fig. 8). The concentration of these compounds that reduced the viability of GMK AH1 cells by 50% (Cytostatic concentration 50%; CC₅₀) was extrapolated from the dose-response curves and their values are shown in Table 2.

![Graphs showing cell viability vs concentration for selected hit compounds](image)

**Fig. 8. Effect of selected hit compounds on viability of GMK AH1 cells.** The cells were growing in 96 well plates for 3 days in the presence of specific concentrations of selected hit compounds specified in Table 2, and then the tetrazolium-based reagent was added and following further incubation for 2 h at 37°C, the absorbance at OD492 nm was recorded. The results are presented as a percentage of absorbance values found in cells incubated with the compound relative to control wells that received no compound. The data shown are means of duplicate determinations. All data points showing statistically significant difference are denoted with asterisks with the following P-values: *P<0.05, **P<0.01, and ***P<0.001.
The CC\textsubscript{50} and IC\textsubscript{50} values of hit compounds were used to calculate the selectivity index (SI) (Table 2). The SI is a CC\textsubscript{50}/IC\textsubscript{50} quotient that is used in antiviral research as a measure of viral specificity of newly discovered antiviral hits. It also suggests whether the use of particular hit compounds is safe or not. The SI value of <1 indicates that the antiviral properties of a hit compound are due to an adverse effect on the cells while SI values >1 suggest the virus-specific inhibitory activity of a hit. Compounds no. 1 and 12 exhibited SI values >5 (Table 2). These are relatively low SI values, however, further optimization of these compounds may help clarify their usefulness as anti-HPIV-2 hits. The same concerns compound 15 that showed SI value of 4.5. On the other hand, compounds no. 4, 5, 8, and 20 exhibited SI values of <1 indicating that their anti-HPIV-2 activities were due to cytostatic properties in GMK AH1 cells.

3.6 Hit compound 5 reduces the size of virus-induced plaques

In spite of cytostatic properties of compound 5 (although not statistically significant; Fig. 8), it appeared that this compound may reduce the size of the HPIV-2 induced plaques in GMK AH1 cells (Fig. 9A-B). Therefore, the size of HPIV-2 plaques induced in the presence of different concentrations of compound 5 was measured (See method in section 2.10). Compound 5 significantly reduced the size of viral plaques at concentrations 40, 8, and 1.6 µM (Fig. 10). At the later concentration (1.6 µM), compound 5 did not show any cytostatic properties (Fig. 8), suggesting that this effect of compound 5 was the virus specific and not due to it cytostatic properties.

![Fig. 9. Reduction of the size of HPIV-2 induced plaques in GMK AH1 cells by compound 5. GMK AH1 cells were infected with the virus in the absence (A) or presence (B) of compound 5 at 40 µM. Following 6 days of incubation at 37°C, the cells were stained with crystal violet to visualize the viral plaques (denoted with arrows). Note that substantially smaller viral plaques developed in the presence of compound 5 as related to untreated control.](image-url)
3.7 Mode of antiviral activity of compound 5

To discriminate whether antiviral activity of compound 5 was due to targeting the virus particles or the cell, the virucidal (See method in section 2.13) and the cell treatment assays were performed. Compound 5 showed no direct virucidal effect on viral particles as the infectious titers of compound-treated and mock (DMSO)-treated virus remained similar, and no statistically significant differences were observed (Table 3). However short treatment (for 4h) of GMK AH1 cells with compound 5 prior to their infection with HPIV-2 was sufficient to reproduce its inhibitory effect on the size of viral plaques (Table 4).

Table 3. Compound 5 showed no direct virucidal (the virus-inactivating) activity

<table>
<thead>
<tr>
<th>Compound concentration (µM)</th>
<th>Infectious titer (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>0 (Control)</td>
<td>3.16x10⁴</td>
</tr>
<tr>
<td>20</td>
<td>2.86x10⁴</td>
</tr>
<tr>
<td>100</td>
<td>2.93x10⁴</td>
</tr>
</tbody>
</table>

The virus (5 x10⁴ PFU) was incubated with indicated concentrations of compound 5 for 15 min at 37°C, and the residual virus infectivity was titrated. Two separate experiments were performed in duplicate, and the results are shown as a mean titer of the virus.

Table 4. GMK AH1 cells pre-treated with compound 5 developed smaller plaques of HPIV-2

<table>
<thead>
<tr>
<th>Compound concentration (µM)</th>
<th>Plaque number Mean ± SD (% of control)</th>
<th>Plaque area (µm²) Mean ± SD (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>309.8 ± 61.3 (100%)</td>
<td>18286 ± 4975 (100%)</td>
</tr>
<tr>
<td>5</td>
<td>274.8 ± 33.9 (88.7%)</td>
<td>16956 ± 5151 (92.7%)</td>
</tr>
<tr>
<td>10</td>
<td>264.6 ± 23.1 (85.4%)</td>
<td>14065 ± 3366 (76.9%)***</td>
</tr>
<tr>
<td>20</td>
<td>255.6 ± 30.2 (82.5%)</td>
<td>13382 ± 3582 (73.2%)***</td>
</tr>
</tbody>
</table>

GMK AH1 cells were pretreated with indicated concentrations of compound 5 for 4 h at 37°C and then the compound was removed and the cells were infected with the virus. Two separate experiments were carried out in triplicate and the data are shown as mean number of viral plaques or a mean area of viral plaques (n=60).

*** Statistically significant difference at P value <0.001
At conditions of this short treatment compound 5 significantly reduced the area of viral plaques at 10 and 20 µM (Table 4) while at conditions of continuous cell treatment throughout the whole period of cell infection and development of viral plaques (6 days) the compound also reduced the size of viral plaques at lower concentrations (Fig. 10).

Fig. 10. Reduction of the size of HPIV-2 induced-plaques in GMK AH1 cells by compound 5. GMK AH1 cells were infected with the virus in the presence of the specific concentration of compound 5. Following 6 days of incubation at 37°C under methylcellulose overlay, the cells were stained with crystal violet to visualize the viral plaques, and then 30 plaques from each compound concentrations (including control wells) were captured using a DC300 digital camera attached to Diavert microscope, and their areas were measured using IM500 software. The results are presented as a percentage of the average area of viral plaques measured in cells incubated with the virus and the compound relative to control wells that received the virus only. The data are shown as averages of 30 determinations. The average area of viral plaques in control wells was 16013.68 µm² (±4082.94). All data points showing statistically significant difference are denoted with asterisks with the following P-values: **P<0.01, and ***P<0.001.
These results suggest that compound 5 targeted the cell and not the virus. To further substantiate this interpretation the time-of-addition assay was performed (See method in section 2.14). In this assay, the test compound was added to cells at different time-points relative to their infection with the virus to investigate which step of the viral life cycle could be affected by the compound. Compound 5 showed similar weak inhibitory effects (not significant) on the virus yield at all time-point examined (Fig. 11). This suggests that compound 5 did not affect any specific step of the viral life cycle, and further supports an interpretation that its inhibitory effect on plaque size was due to targeting of cells. The anti-HPIV-2 activity of compound 5 was found with laboratory strain KS/70-C517, and attempts were made to substantiate it activity with HPIV-2 strains isolated from patients (See method in section 2.3). In spite of the fact that eight different clinical specimens of nasal secretions collected from patients suffering from HPIV-2 diseases were used, the viable virus has not so far been isolated.

![Graph showing antiviral activity of compound 5 added at different steps of HPIV-2 infection of cells.](image)

**Fig. 11.** Antiviral activity of compound 5 added at different steps of HPIV-2 infection of cells. Compound 5 was added to GMK AH1 cells at different time-points relative to infection of cells with $1 \times 10^5$ PFU of the virus. The cells were incubated for 3 days at 37°C and the yield of the infectious virus was titrated in the supernatant. Two separate experiments were performed in duplicate and the mean infectious titer (PFU) is shown.
4. Discussion

The aim of this study was to identify novel antiviral compounds against HPIV-2, a pathogen causing serious croup-like respiratory disease in children. To this end a large collection of compound was screened for antiviral activity in cultures of GMK AH1 cells where the virus fuses these cells thus inducing formation of large syncytia (polykaryocytes). Inhibition of formation of this characteristic CPE served as a basis for identification of novel anti-HPIV-2 candidate compounds. The virus induced polykaryocytes comprised of numerous nuclei and at the electron microscopy level the budding of viral particles from cell plasma membrane was observed thus confirming the virus specificity of the syncytium formation. As reported by others (Howe et al 1967), the assembly and budding of newly formed HPIV-2 virus particles occur at the cell plasma membrane. The virus nucleoprotein positions itself beneath the cytoplasmic side of lipid membrane and viral budding is initiated. In the present study it was found that most of the viral particles had elongated shape rather than the spherical shape with a clearly visible pattern of long nucleocapsid strings. The similar pattern of nucleocapsid arrangement in HPIV-2 virions was observed by others (Howe, 1967). Likewise, the formation of long filamentous HPIV-2 particles in polarized cells was reported (Quizh and Compans, 2000).

Having characterized the virus-induced CPE, an antiviral high throughput screening of diverse compounds was performed based on the inhibition of this specific CPE. The high throughput screening is an established method for identifications of novel drugs including antiviral drugs (Macarron, 2006; Pereira and Williams, 2007; Mayr and Bojanic, 2009). In this kind of massive screening, compounds causing protection of cells from the virus-induced CPE due to their more or less apparent cytostatic properties are frequently identified. Therefore, assessment of anti-proliferative activities of identified hits is of vital importance. This task is performed using cytotoxicity assays that evaluate different features of cell viability such as their metabolism, proliferation, and morphology (Berridge et al., 2005; Crouch et al., 1993; Jones et al., 2001). In contrast to extensive antiviral screening performed to find treatment for the life threatening and deadly viruses such as HIV or hepatitis C virus or with potential pandemic-causing pathogens such as SARS, MERS or Ebola virus (Cinatl et al., 2005; De Clercq, 2012; 2013a; 2013b), HPIV-2 has received little attention. Some antiviral screening assays have been performed with a related virus, i.e., HPIV-3 where hits C5 and C7, identified by Mao et al., (2008), were found to inhibit the virus infection of cell at the step of primary transcription.

In the present study, no compound that could interfere with the virus attachment or neuraminidase activity was identified. HPIV-2 like some other paramyxoviruses and influenza virus possess neuraminidase activity that in influenza virus was found to be a good target for successful antivirals (Von Itzstein et al., 1993). It has been reported that two novel hemagglutinin-neuraminidase inhibitors BCX 2798 and BCX 2855 interfered with HPIV-2 infectivity (Alymova et al., 2004). Since, the virus-specific neuraminidase does not occur in human body, recombinant sialidase enzyme that cleaves off sialic acid from the cellular receptor for viruses was successfully used for prevention of HPIV infection in cultured cells and in laboratory animals (Moscona et al., 2010).
In this project, several hits, i.e., compounds 1, 12, and 15 that inhibited HPIV-2 infection of GMK AH1 cells with IC_{50} values of 20 µM while showing the 50% cytotoxicity (CC_{50}) in GMK AH1 cells at 90 µM (compound 15) and >100 µM (compounds 1 and 12) were identified. Hence, the calculated selectivity index values for compounds 1, 12, and 15 were >5, >5, and 4.5 respectively.

The selectivity index i.e., the range between antiviral and cytotoxic/cytostatic activity of a hit compound is an important feature in pre-clinical evaluation of antiviral drugs and in clinical trials where it is referred to as therapeutic index (Muller and Milton., 2012). In antiviral practice compounds that shows selectivity index of ≥10 are considered to have the virus-specific mechanism of antiviral activity. Hence, compounds 1, 12, and 15 might have inhibited HPIV-2 infection of cells due to their non-apparent effect on cellular metabolism.

Interestingly, several compounds approved for antiviral treatment are known to possess cellular rather than viral targets. An example of that is the antiviral drug ribavirin that is used in therapy of a number of infections caused by human RNA viruses including HPIV-2 virus (Kalimuddin et al., 2013). This drug is an inhibitor of cellular inosine monophosphate dehydrogenase that reduces cellular pool of guanosine nucleotide required for replication of viral RNA (Leyssen et al., 2005). Another example of antiviral that targets cellular components are small molecule activators of cellular ribonuclease L, an enzyme that is required for interferon response against some RNA viruses (Thakur et al., 2007). These inhibitors, identified in screening assay, activated RNAse L thus inducing interferon and blocking infection by a number of viruses including HPIV-3 (Thakur et al., 2007). Although at present it is difficult to speculate what cellular components could be targeted by compounds 1, 12, and 15, an increasing number of data indicates that different viruses including paramyxoviruses may selectively modulate various cellular pathways. Thus, RSV was shown to selectively activate epidermal growth factor receptor pathway in order to prolong survival of the virus-infected lung epithelial cells (Monick et al., 2005).

Another HPIV-2 hit compound identified in the present study was compound 5 that manifested its antiviral activity by reducing the area of viral plaques at non-cytotoxic concentrations. Interestingly, compound 5 did not inhibit initial infection of cells and the yield of infectious progeny virus produced in GMK AH1 cells. Furthermore, this compound showed no direct virucidal (the virus-inactivating) activity against HPIV-2 particles suggesting that it did not bind directly to the viral particles thus blocking them or causing their disruption. Some candidate antivirals such as PG545 have shown a direct virucidal activity against the related paramyxovirus RSV (Lundin et al., 2012). The time-of-addition assay, which is routinely used in antiviral research to identify the step of viral life cycle affected by the hit compound (Lundin et al., 2013), revealed that compound 5 did not affect any specific step of viral life cycle since the amounts of newly produced virus was not reduced and remained the same irrespectively of the time of compound addition. Instead, compound 5 exerted the viral plaque size-reducing activity by targeting the cell as a relatively short pre-treatment of cells with this compound prior to viral infection was sufficient for the plaque size reduction.
Some candidate antiviral compounds such as PI88 (Nyberg et al., 2004) are known to reduce the size of the viral plaques by entering a narrow space between infected and non-infected cells where this compounds blocked the viral attachment components. Compound 5 is unlikely to interfere with the HPIV-2 attachment (HN) or entry (F) proteins since the time-of-addition assay revealed no interference when this compound was added to cells at the beginning of their infection with virus. The reduction of plaque size by compound 5 may suggest its interference with the cell-to-cell spread of the virus.

The viruses including HPIV-2 can spread to non-infected cells by the virus released from apical membrane of infected cells or by the direct cell-to-cell spread, i.e., the viral transmission from lateral membrane of infected cell to adhered non-infected cell. Paramyxoviruses such as HPIV-2 (Schaap-Nutt et al., 2010) or RSV (Zhang et al., 2002) target ciliated cells of human respiratory epithelium where the virus spread occurs mainly via the viral particles released into airways from the apical surface of infected cells. However, these viruses can produce syncytia of cells in the airway epithelium of infected patients (Johnson et al., 2007) indicating the virus can also spread by direct cell-to-cell contact. HPIV-2 and many other paramyxoviruses are known to cause syncytial plaques in cultures of infected cells, and the viral F (fusion) protein expressed either on the surface of the infected cell or on the surface of viral particles can mediate fusion between membranes of infected and non-infected cells thus producing large syncytia (polykaryocytes).

However as mentioned earlier interference of compound 5 with the syncytium-forming activity of HPIV-2 is unlikely due to the fact that the viral plaques, the size of which was reduced by compound 5, were still of syncytial phenotype (see Fig. 6). Therefore, compound 5 may affect certain cellular signaling pathways of importance for the viral life cycle that could slow down the viral replication and production of viral components so the cell-to-cell spread of the virus is delayed and thus the viral plaques are smaller.

Replication of viruses in cells is strongly dependent on certain cellular components or metabolic pathways which are required for completion of viral life cycle including production of infectious progeny viruses. Viruses selectively modulate expression of cellular genes causing down-regulation of many cellular proteins and up-regulation of essential protein components required for replication of particular virus. The same concerns paramyxoviruses where host cell genes required for replication of HPIV-3 have been identified with a help of screening of small interfering RNAs (siRNA) (Panda et al., 2011). Selective up-and down-regulation of specific viral genes by paramyxoviruses frequently result in modulation of host immune response (Parks and Alexander-Miller, 2013). One cannot exclude that compound 5 caused down- or up-regulation of genes required for replication of HPIV-2. Modification of expression of such genes could be tolerated by a cell with no adverse effects on its viability detected in cell proliferation assay. However, such alteration in a cellular gene expression may adversely affect the virus replication.
Altogether several hit compounds that moderately affected infectivity of HPIV-2 in GMK AH1 cells were identified by screening of the large diversity collection of compounds. Screening of the same collection of compounds against RSV infection in HEp-2 identified epidermal growth factor receptor tyrosine kinase inhibitor AG-1478 as a promising anti-RSV hit compound (Lundin et al., unpublished observation). This finding seems to be in line with previously reported activation of cellular epidermal growth factor receptor by RSV (Monick et al., 2005). This hit was not identified in the present screening for HPIV-2. This indicates that these two related paramyxoviruses may show subtle differences in regard to their requirement for activation of specific cellular genes. Anyhow, further detailed studies are required to clarify the mode of antiviral activity of compound 5.
5. Ethical aspects and impact on the society

This part of the project does not include study on experimental animals. Isolation of clinical strains of the virus from specimens of patients is a part of routine diagnosis performed at the Department of Clinical Virology, Sahlgrenska Hospital/University of Gothenburg.

Viral respiratory infections are very common in humans, and every person suffer from those infections at least once a year. However, except for influenza virus no specific vaccines or antiviral is available and the aim of the project is to develop antiviral drug against one of the viruses that causes respiratory infections. Such antiviral may decrease respiratory discomfort in sick people and may reduce economic losses due to the work absence.
6. Future perspectives

As a part of a long-term project aiming at the development of novel antivirals against acute viral respiratory infections, the most promising anti-HPIV-2 hit compound 5 will be subjected to further studies and development. This will include chemical optimization of compound 5 by synthesis of a number of its analogues. This task will be performed in collaboration with Dr. Nina Kann (Chalmers University of Technology, Gothenburg). The synthesized analogues of compound 5 will be subjected to evaluation of their antiviral activity and cytotoxic properties, the aim being to identify analogues with improved antiviral activity and decreased cytotoxicity. Such lead compound is then subjected to detailed elucidation of the mechanism of its antiviral activity according to the strategy described previously (Lundin et al., 2013). This part of the project will be carried out in collaboration with Dr. Richard Kingston (The University of Auckland, New Zealand) who performs research on structure and function of paramyxovirus polymerase proteins (Kingston et al., 2004). The anti-HPIV-2 activity of the lead compound will also be evaluated in well-differentiated cultures of human airway epithelium to verify biological significance of developed lead compound as previously described for anti-coronavirus compound K22 (Lundin et al., 2014).
7. References


