

## Chapter 1.4

# INHIBITORS OF RESPIRATORY VIRUSES

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**Abstract:** Viral respiratory infections are caused by a variety of different virus species such as human rhinovirus, influenza virus, parainfluenza and respiratory syncytial virus. As the life cycles of these viruses are quite different, the development of a general treatment for respiratory infections seems impossible. For specific viruses however significant progress has been achieved, although only for influenza virus antiviral medication has reached the market. We present different approaches to inhibit respiratory viruses with a focus on human rhinoviruses, the most frequent cause of common cold.

## 1. INTRODUCTION

### 1.1 Main Approaches

There are many viruses which cause diseases in the respiratory tract of humans and mammals. A variety of different virus families such as rhinovirus, influenza virus, parainfluenza virus, respiratory syncytial virus (RSV), coronaviruses and adenovirus are responsible for more than 95% of all human respiratory infections. The remaining 5% are due to bacterial infections. Typical symptoms of respiratory infections develop one to two days after viral inoculation and include nasal discharge, sneezing, sore throat, cough, headache and general weakness. In severity, the symptoms may vary from a mild “common cold” to a severe influenza virus infection, but they still make some 60 million people seek medical advice every year in the United States alone (Bertino 2002). The National Centre for Health

Statistics estimated that in 1996 about 62 million cases of viral respiratory tract infections (VRTI) required medical attention or resulted in restricted activity in the United States. Roughly one in six respiratory infections leads to a doctor's office visit and up to 50% of these visits result in an antibiotic prescription, which mainly treats secondary infections but fails to tackle the primary viral infection (Bertino, 2002). The market value relating to respiratory tract infections is illustrated by the fact that in 1998 more antibiotic prescriptions were written for presumed VRTIs than for bacterial infections, at a cost of approximately \$ 726 million (Gonzales *et al.*, 2001).

The need for an effective treatment of VRTIs is obvious. In 1998, people in the United States spent around \$ 5 billion on over-the-counter products that relieve symptoms of the common cold. This corresponds to a rise by more than 50% as compared to 1995, when the amount spent was \$ 3.3 billion.

As viral respiratory infections are caused by a wide range of different viruses that require an equally great variety of treatments, establishing successful causative treatment is a challenging task. We will provide an overview on treatment approaches and discuss problems and potential solutions for successful therapy.

## **2. RESPIRATORY VIRUSES**

### **2.1 Epidemiology**

Viral respiratory tract infections are caused by a heterogeneous group of viruses of different genera. The relative proportions of these viruses vary and depend on several factors such as season, age but also viral sampling and detection technique. About 30-50% of all infections – generally termed as “common cold” – are caused by rhinoviruses of the picornavirus group. This makes common cold the most widespread acute disease in individuals (Monto *et al.*, 1993, 2002a; Makela *et al.*, 1998). Approximately 5-15% of all viral respiratory infections are caused by influenza virus, which makes them more severe and sometimes lethal. Most people recover from the illness; however, the Centers for Disease Control and Prevention (CDC) estimates that in the United States an average of 36,000 persons die from influenza and its complications every year, most of them elderly people. Respiratory syncytial virus (RSV) is the main cause of infant bronchiolitis and pneumonia. The virus is ubiquitous, highly infectious and reaches epidemic proportions every year during the winter months. In the total population,

approximately 5% of all VRTI cases are caused by RSV. Parainfluenza virus can cause upper and lower respiratory tract infections in both adults and children (3%). Coronaviruses are found in 7-18% of all adults suffering from VRTIs (Larson *et al.*, 1980; Nicholson *et al.*, 1997). Single study estimated that human coronaviruses account for up to 35% of cases of upper respiratory illness. While it is clear that human coronavirus can play an important role in respiratory outbreaks a much lower frequency is found in most studies (Vabret *et al.*, 2003; Louie *et al.*, 2005). The two remaining virus families, i.e. adenovirus and enterovirus, account for minor shares of respiratory infections. The fact that up to 30% of VRTIs are not assigned to a certain virus species is probably due to suboptimal sampling and detection methods in some cases (Heikkinen *et al.*, 2003).

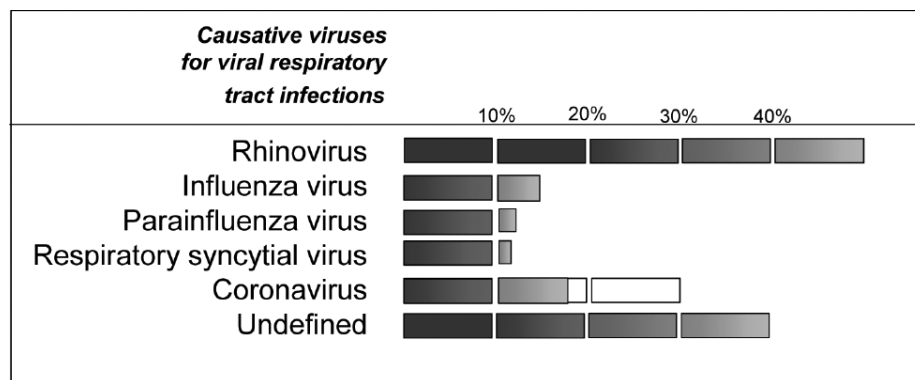


Figure 1. Causative viruses for viral respiratory tract infections (compiled from Monto 2002a and Gwaltney *et al.*, 1966).

An analysis of available epidemiology data has shown a distinct and consistent seasonal pattern in the occurrence of respiratory viruses (Monto 2002a). In temperate regions of the northern hemisphere, rhinoviruses account for up to 80% of all viruses circulating in early autumn (Arruda *et al.*, 1997; Makela *et al.*, 1998). In some years and some geographic areas, spring is an even more dangerous time for rhinovirus transmission. Although overall rates of respiratory diseases are lower in summer, rhinoviruses are the type of virus that is most frequently isolated at that time of the year. In winter, other viral agents, among them influenza viruses, parainfluenza virus and respiratory syncytial virus, predominate in the northern hemisphere (Monto 2002b).

## 2.2 Diagnostic Problem

From a clinical point of view, ascertaining upper respiratory tract virus infection is difficult, because many respiratory viruses cause similar symptoms. Temperatures above 37.8°C in the initial stage of the disease indicate influenza rather than other virus infections. Early identification of respiratory viruses is essential for effective diagnosis and patient management, as only for influenza virus causative treatment is available. Methods for identifying viruses include viral culture, antigen detection and highly sensitive molecular biology techniques based on polymerase chain reaction (PCR). Isolating viruses in cell culture is considered the gold standard but of limited relevance in clinical practice because it is too slow. Several antigen detection tests are available for diagnosing influenza A and B, parainfluenza, RSV and adenoviruses (Kim *et al.* 1983; Waris *et al.*, 1988; Nikkari *et al.*, 1989) though they are not widely used (Steininger *et al.*, 2001, 2002). Due to the high number of human rhinovirus (HRV) serotypes, detecting these viruses by immunological methods is not easy although PCR methods are available. A main disadvantage of the latter is the fact that they do not discriminate between infective viruses but rather detect the viral genome.

## 3. INHIBITION OF RESPIRATORY VIRUSES

Currently, we lack a general treatment that addresses the underlying causes of viral respiratory tract infections, i.e. the virus infection.

In principle, antiviral drugs can be designed to either target a viral or a cellular protein. Targeting specific proteins of viruses basically has the advantage of being less toxic for cells with a narrow antiviral spectrum, while targeting cellular molecules might yield compounds with a broader antiviral activity. In the first case, the likelihood of generating drug-insensitive mutants is high, while this risk is unlikely in the latter case. The concept was proved for both strategies, e.g. in infections with human rhinoviruses. One example is the successful use of inhibitors targeting the viral proteinase 3C in HRV. A cellular approach was taken by the use of interferon to stimulate cellular defense against rhinovirus. For a thorough review of the development of antiviral strategies see a recent review by DeClerq (2004).

### 3.1 Rhinoviruses

Rhinovirus infections may account for up to one third of all “common colds”. The virus family consists of more than 100 serotypes, is ubiquitous and can cause repeated episodes of infection throughout an individual’s life time. Infection in otherwise healthy persons is unpleasant though usually self-limited. However, certain populations may be predisposed to severe manifestations, including bronchiolitis and pneumonia in infants and exacerbations of pre-existing airway disease in persons with chronic obstructive lung disease, asthma and cystic fibrosis (Hayden 2004).

#### 3.1.1 Pathogenesis

The pathogenesis of a rhinovirus infection was elucidated by many studies of volunteers infected with rhinoviruses (Gwaltney *et al.*, 1966, 1975, 1977, 1978). Infection begins with the deposition of viral particles in the anterior nasal mucosa or in the eye, from where the virus can be transported to the nose via the lacrimal duct. At the mucosal surface, virions attach themselves to cellular receptors. Based on receptor specificity, rhinoviruses can be divided into two groups: the viruses belonging to the “major group” attach themselves to the cellular adhesion molecule-1 (ICAM-1), whereas “minor group” viruses bind to the LDL receptor or LDL-related proteins (Greve *et al.*, 1989; Hofer *et al.*, 1994). The serotype HRV 89 is reported to bind to heparan sulfate proteoglycan (Vlasak *et al.*, 2005). Following internalization and uncoating, the viral plus-strand RNA is translated into one large polyprotein, which is proteolytically processed by two viral proteases – 2A and 3C – into the individual viral proteins. Replication is mediated via a minus-strand RNA intermediate and catalyzed by the viral 3D polymerase. Resulting RNA molecules can then be used for translating viral proteins or are packaged into viral particles in a late phase of the infection. Viral proteinases specifically cleaving factors such as the eukaryotic initiation factor 4G required for cellular cap-dependent translation turn the host cell into a virus producing machine (host cell shutoff) (Etchison *et al.*, 1987). The viral mRNA is translated via a cap-independent mechanism involving direct binding of ribosomal subunits to internal ribosomal entry sites, which facilitates virus production. In addition, this host cell shutoff limits the defense response to the viral infection e.g., the interferon response, as cellular translation is inhibited (Weber *et al.*, 2004). Viral release is mediated via destruction of the host cell. Surprisingly and differently to patients infected with influenza virus, individuals infected with rhinoviruses show no major tissue destruction in nasal biopsies. This observation suggests that the clinical symptoms of rhinovirus infection might not be due to cell

destruction mediated by virus replication (cytopathic effect) but are primarily caused by the immune response of the host. Several inflammatory mediators can be found in the nasal secretion of patients with common cold, e.g. interleukin 1, 6 and 8, histamines, leukotriens and kinins. Interleukin 6 and 8 levels correlate with the severity of symptoms. In contrast to the pro-inflammatory reaction, rhinovirus can even down-modulate an appropriate immune responses by inducing immunosuppressive cytokine interleukin 10 as was shown in monocytes (Stockl *et al.*, 1999). However, the immunological host response to rhinovirus infection is far from being fully explored. On average, colds take one week, although 25% of all cases last longer. Viral shedding can be observed up to three weeks, though the risk of transmission diminishes after three days (D'Alessio *et al.*, 1976).

### **3.1.2 Secondary infections**

With the help of modern PCR techniques, rhinoviruses have been detected more frequently at sites distant from the primary infection. This led to a greater appreciation of the role this pathogen plays in upper and lower respiratory tract disease. Specifically, rhinoviruses are supposed to be associated with otitis media (Pitkaranta *et al.*, 1998, 1999), sinusitis, asthma exacerbations, chronic obstructive pulmonary diseases and lower respiratory infections in elderly, neonates and in immunocompromised individuals (Greenberg 2003). In children, the most common bacterial complication is otitis media, which can occur in up to 20% of all children with viral upper respiratory tract infections. Using PCR techniques, various viruses were detected in middle ear fluid, suggesting a causative involvement of these viruses (Pitkaranta *et al.*, 1997, 1998, 1999, 2002). HRVs were also detected in the lower respiratory tract (Papadopoulos *et al.*, 1999; Mosser *et al.*, 2002, 2005).

### **3.1.3 Antiviral Agents**

Even though the common cold and its complications are medically important, efforts to find causative treatment have been rather futile. At present, there are no approved antiviral agents for treating HRV infection. Several treatment strategies were evaluated, albeit with limited success so far.

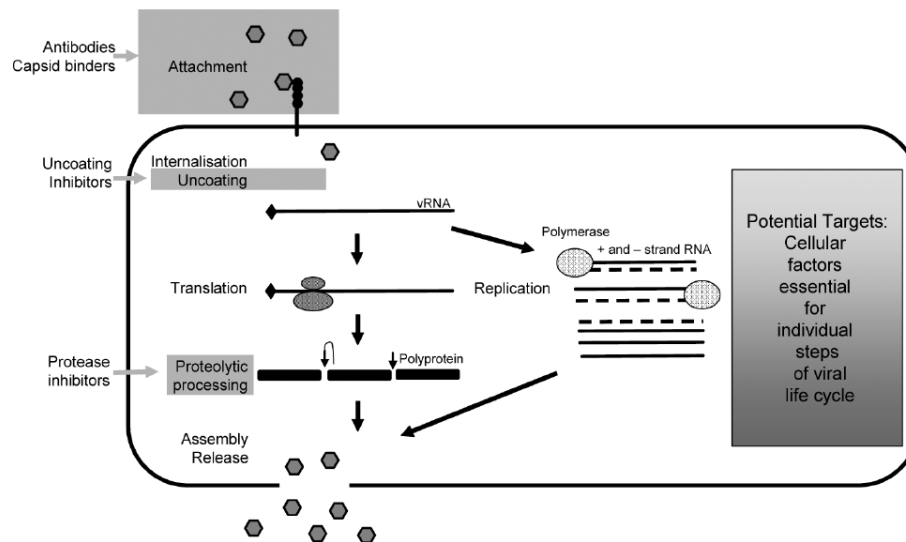


Figure 2. Rhinovirus life cycle. Current approaches for viral inhibition are indicated.

In the seventies of the last century Isaacs and Lindenmann reported a factor that could induce a virus resistant state that was termed “interferon” (Isaacs *et al.*, 1957a, 1957b; Lindenmann *et al.*, 1957). Interferon can trigger an antiviral response in the host cell based on activation of expression of more than 300 interferon induced genes that have antiviral, antiproliferative or immunomodulatory functions. The best studied interferon induced antiviral proteins are the 2’5’ oligo adenylate synthetase, protein kinase R and the MX GTPases. From experiments in knock-out mice it became evident that also additional pathways exist (Zhou *et al.*, 1999). Although the importance of the interferon response to viral infections is clear, their mode of action is still incompletely understood (Weber *et al.*, 2004). Based on the broad induction of a cellular antiviral response interferon were used as “general” antiviral medication. In several studies, it proved to be an effective prophylactic but was associated with significant clinical and histopathological signs of nasal irritation (Hayden *et al.*, 1986). However, treatment with interferon neither cured experimentally induced nor naturally occurring colds (Farr *et al.*, 1984; Hayden *et al.*, 1984, 1988b; Turner *et al.*, 1986; Sperber *et al.*, 1989). Interferons are an example of molecules targeting a cellular rather than a viral function.

As the majority of HRVs bind to the intercellular adhesion molecule-1 (ICAM-1) as the receptor, strategies using soluble decoy receptors were developed to block attachment (Marlin *et al.*, 1990). Studies with such receptors such as “tremacamra” showed that it reduced the severity of the disease though the effect was modest (Turner *et al.*, 1999).

Monoclonal antibodies against ICAM-1 were used to inhibit the attachment of viruses to their cognate receptors. The antibody delayed the onset of HRV-induced colds in human volunteers but did not reduce the incidence of common cold in a clinical trial (Hayden *et al.*, 1988a). A further development of this receptor blocking approach is the generation of humanized anti-ICAM antibodies (Luo *et al.*, 2003) with higher affinity or the construction of multivalent anti-ICAM-1 antibody Fab fusion proteins with higher avidity (Charles *et al.*, 2003; Fang *et al.*, 2004).

Detailed analysis of virus-cell interaction led to the identification of another group of inhibitory substances, i.e. capsid-binding substances. Several compounds were identified (Rosenwirth *et al.*, 1995; Oren *et al.*, 1996) or developed over the last years, e.g. WIN-compounds such as disoxaril (McKinlay 1985; Andries *et al.*, 1992; Mallamo *et al.*, 1992) or pirodavir. However, in clinical trials, pirodavir was efficient from a prophylactic but not from a therapeutic point of view (Hayden *et al.*, 1995). This might be attributed to poor pharmacokinetics. Currently new molecules related to pirodavir are being evaluated (Barnard *et al.*, 2004). Pleconaril is the capsid-binding substance that has been tested most extensively. It is effective against a variety of rhino- and enteroviruses, orally bioavailable and was studied in more than 5,000 patients in clinical trials. If pleconaril treatment is started early, it reduces the duration and severity of the disease (Hayden *et al.*, 2002, 2003a Viropharma 2002). However, the US Food and Drug Administration did not approve pleconaril (Picovir™) in May 2002, mainly because of the observed induction of cytochrome P450 3A enzymes, which metabolize a variety of drugs, e.g. oral contraceptives. A new intranasal formulation of pleconaril is currently developed by Schering-Plough. Intranasal application should enable a more efficient delivery of the drug to the site of infection than the oral formulation, while limiting its systemic exposure and thereby minimizing the risk of drug interactions. Such other capsid-binding substances as BTA 798 are in the stage of early preclinical development.

Enviroxime is a compound inhibiting HRV multiplication. It targets a step in the RNA replication complex that depends on the availability of the viral proteins 3A and 3AB (Heinz *et al.*, 1995; Brown-Augsburger *et al.*,



1999). Similarly to 3C proteinase inhibitors, this class of compounds can be applied in tissue culture several hours after inoculation without loss of activity, as it targets a later step in the viral life cycle. Intolerance to oral dosing, poor pharmacokinetics, undesirable toxic side effects and modest benefits after intranasal administration hampered further development (Phillipotts *et al.*, 1981, 1983; Hayden *et al.*, 1982; Levandowski *et al.*, 1982; Miller *et al.*, 1985). However, derivatives are currently under preclinical evaluation.

Identification and molecular characterization of essential viral proteinases in HRV paved the way to the development of protease inhibitors to combat rhinoviral infection (Libby *et al.*, 1988; Sommergruber *et al.*, 1989).

The two viral proteinases 2A and 3C are both essential for virus multiplication. These enzymes are involved in processing the viral poly-protein into single viral proteins. Mutations in the active sites of these proteinases lead to total inhibition of virus multiplication. Structurally, both proteases are related to trypsin-like serine proteases. Functionally, however, the active site amino acid is replaced by a cysteine residue. Sequence comparisons among several HRV serotypes have demonstrated a high degree of homology among amino acid residues involved in the active site of the molecule (Seipelt *et al.*, 1999). As these proteases are not closely related to such other cellular cysteine proteases as caspases, they are an attractive target for drug discovery (Matthews *et al.*, 1994).

Determination of the three-dimensional structure of the 3C protease and subsequent modeling led to the identification and development of specific peptide aldehyde inhibitors (Matthews *et al.*, 1994; Patick *et al.*, 1999; Kaiser *et al.*, 2000). AG-7088 is an irreversible peptidomimetic inhibitor with broad activity against several HRV serotypes, HRV clinical isolates and related picornaviruses *in vitro* (Patick *et al.*, 1999; Kaiser *et al.*, 2000; Binford *et al.*, 2005). Importantly, AG-7088 is also effective when added in cell culture post infection, which might be an advantage compared to capsid-binding substances. Data from *in vitro* and phase I and II studies suggest that AG-7088 is an effective and safe inhibitor of HRV replication (Hsyu *et al.*, 2002; Hayden *et al.*, 2003b). In phase II trials, a nasal spray of AG-7088 (rupintrivir) significantly reduced total cold symptoms and was well tolerated. Prophylaxis lowered the share of subjects with positive viral cultures and viral titers. Surprisingly, it did not reduce the frequency of colds, nor did early treatment decrease the frequency of the disease though it lowered the severity of daily symptoms. As rupintrivir was not able to

significantly effect virus reduction and moderate disease severity in subsequent natural infection studies in patients, Agouron Pharmaceuticals terminated the development. Similarly, Eli Lilly designed a compound targeting the human rhinovirus 3C protease (LY 338387) but no recent development was reported. In addition, an orally bioavailable inhibitor of HRV 3C protease was identified. A Phase I trial showed bioavailability and lack of toxicity (Patick *et al.*, 2005). Unfortunately, the development of this inhibitor for clinical use is not continued. It should be noted that 3C proteinase inhibitors are effective against a variety of HRV serotypes, as most of the amino acids critical for binding the protease inhibitor are conserved (Binford *et al.*, 2005).

In principle, the 2A protease of HRV is also an attractive target for therapeutic intervention. The protease activity is essential for virus multiplication; it is highly specific to its cognate cleavage site. In the life cycle, the protease is directly involved in cleaving the translation factor eIF4G, which shuts off host cell translation. So far, no specific inhibitors for the HRV 2 protease are available. Recently, we could show that the methylated form of a commonly used caspase inhibitor, zVAD.fmk, inhibits HRV2 2A protease *in vitro* and in cell culture, leading to an inhibition of viral multiplication (Deszcz *et al.*, 2004). However, this is rather an undesired side effect discovered in experiments aimed at specific inhibition of caspases. Fluoromethyl ketone derivatized peptides used as inhibitors of caspases are usually employed as methyl-esters to facilitate cell permeation. Inside the cell endogenous esterases cause the demethylation of the inhibitors. We could show that the methylated form specifically inhibits 2A activity, whereas the de-methylated form does not. This is in good agreement with substrate requirements for the 2A protease (Skern *et al.*, 1991; Sommergruber *et al.*, 1992). However, these experiments clearly show that care must be taken when a “specific” inhibitor is used and exemplify, that inhibition of 2A protease does indeed lead to the block of HRV multiplication.

#### 3.1.4 Resistance Problem

A major problem associated with common antiviral drugs targeting specific viral functions is the emergence of resistant escape mutants due to selective pressure and the high error rate in viral replication. Human rhinoviruses, as other RNA viruses, evolve as complex distributions of mutants termed viral quasispecies (Domingo *et al.*, 2005). Inhibition of viral enzymes favours the selective advantage of some viral subpopulations over others (Vignuzzi *et al.*, 2005).

Although patients treated with pleconaril show a rapid decrease of viral RNA, they occasionally continued to have positive cultures on study day 6 or later, though on a low level. In a study, viruses with at least tenfold reduced susceptibility to pleconaril were found in 10% of all patients who received this drug (Hayden *et al.*, 2003a). The genotype was evaluated for picornaviruses with reduced susceptibility to pleconaril following exposure to the drug. In all cases examined, the molecular basis of the reduced susceptibility involved amino acid changes in the drug-binding pocket of capsid protein VP1. Similarly, resistant mutants can be found when treatment is done with rupintrivir or other anti-rhinoviral substances (Heinz *et al.*, 1996; Nikolova *et al.*, 2003).

To combat resistance, two main strategies are available: (i) using combinations of antiviral substances with a different mode of action to increase the selective pressure on the virus or (ii) targeting cellular rather than viral proteins.

So far, several combinations of antiviral and anti-inflammatory agents were clinically tested, albeit with limited success (Gwaltney 1992; Sperber *et al.*, 1992; Stone *et al.*, 1992). Targeting cellular functions is difficult based on our limited understanding of the complex host virus interactions. Identification of essential cellular proteins for viral multiplication and a more complete picture regarding the antiviral capabilities of host cells will allow targeting these processes for future therapeutic strategies.

### 3.1.5 Unspecific agents

Self-medication includes decongestants (alpha-adrenergic agonists) such as pseudoephedrine (Sperber *et al.*, 2000) and phenylpropanolamine or anticholinergic agents such as ipratropium bromide (Winther *et al.*, 2001). These compounds are moderately active in relieving nasal obstructions and rhinorrhea. Nonsteroidal anti-inflammatory drugs such as ibuprofen significantly reduce fever, sneezing and headache associated with colds (Winther *et al.*, 2001). Antitussives, expectorants, mucolytics, antihistamine-decongestant combinations and other over-the-counter medicines are similar to placebo in relieving acute cough associated with upper respiratory tract infections (Smith *et al.*, 1993).

For several years, zinc has been considered a possible treatment of many illnesses (Hill *et al.*, 1987; Prasad *et al.*, 1989, 2002; Doerr *et al.*, 1997), amongst them respiratory tract infections. Zn salts are believed to have

immunomodulatory effects and inhibit rhinovirus replication *in vitro*, possibly by inhibition of the viral 3C proteinase (Cordingley *et al.*, 1989) (Korant *et al.*, 1974; Geist *et al.*, 1987). Several trials have evaluated various preparations for treating respiratory illnesses (Farr *et al.*, 1987). Based on these results, one can conclude that benefit is lacking (Jackson *et al.*, 1997, 2000; Belongia *et al.*, 2001). The role of vitamin C in the prevention and treatment of common cold has been a subject of controversy for many years. However, a meta-analysis of studies reveals no major benefit for the public, except for minor subgroups (Douglas *et al.*, 2005). Studies with echinacea pose the problem of its varied composition in different preparations, as it stems from a natural product. Therefore, study results are not comparable and inconclusive. However, when compared to placebo, these supplements did not show a strong benefit for the patients (Grimm *et al.*, 1999; Turner *et al.*, 2000; Schroeder *et al.*, 2004). Perhaps the easiest though systematically not well-examined procedure with a soothing effect is inhalation of heated humidified air. This was shown to reduce symptoms but had no influence on viral shedding (Singh 2004).

To a limited extent also prevention of rhinovirus infection was investigated experimentally. As rhinoviruses are sensitive to acid pH values, this property can be used to reduce person-to-person transmission. Disinfecting tissues with various compounds such as citric acid, malic acid and sodium lauryl sulfate have been used under experimental conditions but not in natural settings (Hayden *et al.*, 1985a; Hayden *et al.*, 1985b; Dick *et al.*, 1986).

### 3.2 Influenza

Amantidine is a ion channel blocker and has been used to treat and prevent influenza A for many years (1970). It blocks the viral M2 ion channel and thus prevents acidification and uncoating of the virus. Influenza B types do not have an M protein but have an NB protein which is resistant to amantadine. In the United States, rimantidine is frequently used because of its lesser side effects. In clinical trials, amantidine reduced the duration of the disease, though side effects and the frequent emergence of resistant viruses could be observed (Dolin *et al.*, 1982; Hayden *et al.*, 1989, 1991; Sweet *et al.*, 1991).

Based on the crystal structure of the influenza virus neuraminidase, zanamivir was developed as a specific inhibitor of this enzyme. Zanamivir is applied as inhalation and has been shown to be efficient and safe for treating influenza virus. Moreover, it is licensed for clinical use. Oseltamivir is an

orally available prodrug and is also licensed. When treatment is initiated within 48 h after infection, the duration of the disease is reduced by 1 to 2 days. Since these drugs target influenza virus only, they do not protect patients against infections caused by other viruses involved in viral respiratory infections. This means, within this limited time frame, influenza has to be correctly diagnosed versus rhinovirus and other viral respiratory infections. Further developments include the cyclopentane peramivir (BCX-1812, RWJ-270201), a highly selective inhibitor of influenza A and B virus neuraminidases and a potent inhibitor of influenza A and B virus replication in cell culture (Smee *et al.*, 2001). In clinical trials with patients experimentally infected with influenza A or B viruses, oral treatment with peramivir significantly reduced nasal wash virus titers with no adverse effects. Phase III clinical trials are underway (Sidwell *et al.*, 2002).

Originally, resistant mutants against zanamivir and oseltamivir were rare. However, new results in children show the emergence of resistance in up to 18% (Kiso *et al.*, 2004).

For a more detailed discussion of neuraminidase inhibitors see chapter 1.5.

### 3.3 Coronaviruses

Human coronavirus infections such as 229 E were not considered serious enough to be controlled by vaccination or antiviral treatment. This view changed rapidly with the emergence of SARS, which has been associated with a newly discovered coronavirus (SARS-CoV) (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003). Therapeutic strategies against SARS-CoV are described in chapter 3.1.

### 3.4 RSV

Research for an efficient treatment and vaccine against RSV was of limited success (Maggon *et al.*, 2004). Palivizumab is a humanized IgG1 monoclonal antibody that binds to the F-protein of RSV. It consists of human (95%) and murine (5%) antibody sequences and thus has a low rate of inducing immunogenic reactions. The drug exhibits neutralizing and fusion-inhibitory activity against RSV. The protection in children is transient and has to be repeated during the RSV season. Each immunization protects a baby for about 30 days, so a new vaccination is needed each month during the respiratory syncytial virus (RSV) season.

## 4. FUTURE APPROACHES

### 4.1 Antiviral molecules with unknown function

Our group has found that pyrrolidine dithiocarbamate (PDTC), a commonly used inhibitor of the transcription factor NF- $\kappa$ B, exerts a strong antiviral effect on the multiplication of human rhinovirus and poliovirus (Gaudernak *et al.*, 2002). Other groups recently confirmed these results with such other picornaviruses as the coxsackievirus (Si *et al.*, 2005). Interestingly, the antiviral property is not confined to the picornavirus family, as we and others have shown that PDTC is also active against influenza viruses, having a very different life cycle (Grassauer *et al.*, unpublished, Uchide *et al.*, 2002, 2005). However, other viruses such as tick-borne encephalitis virus are not inhibited by PDTC.

Currently, the striking property of PDTC and functionally related compounds is under investigation. We have shown so far that PDTC does not affect early steps in HRV infection such as virus attachment, internalization and uncoating. However, polyprotein processing and replication are severely impaired when PDTC is present (Krenn *et al.*, 2005).

What is the mechanistic basis of this inhibition?

PDTC is widely used as a specific inhibitor of the transcription factor NF- $\kappa$ B in eukaryotic cells. The molecular mechanism by which PDTC inhibits NF- $\kappa$ B is not yet elucidated and results obtained by different groups are contradictory. Depending on which cellular system is used, both antioxidative properties and prooxidative effects of PDTC have been described. Furthermore, inhibition of the ubiquitin-ligase system was discussed as being involved in the inhibition of NF- $\kappa$ B. An important question is whether the NF- $\kappa$ B inhibitory property of PDTC is related to its antiviral effect. Regarding this aspect, we believe that the inhibition of NF- $\kappa$ B is not important during rhinoviral infection. The transcription factor NF- $\kappa$ B is activated during picornaviral infection. However, as the viral proteinase 2A leads to a fast shut off of host cell transcription during infection, it seems unlikely that NF- $\kappa$ B - mediated genes have a major effect on the replication of HRVs, because the corresponding mRNAs cannot be translated. Certain NF- $\kappa$ B-induced genes may translate via a cap-independent mechanism, though experimental evidence for this theory is lacking. We have also shown that replication of HRV in cells, in which NF- $\kappa$ B is constitutively inactivated by overexpression of the cellular inhibitor I $\kappa$ B, cannot be distinguished from controls with functional NF- $\kappa$ B pathways. Other inhibitors such as aspirin also fail to exert an antiviral effect against HRV. From these data we conclude that the widely known NF- $\kappa$ B inhibitory

property of PDTC is not important for the antiviral effect in HRV. However, in influenza virus the situation might be different.

Mechanistically, we have shown that metal ions such as copper and zinc ions are involved in the antiviral effect of PDTC (Krenn *et al.*, 2005). This is an agreement with recent reports obtained for the inhibition of coxsackievirus (van Kuppeveld F.J, pers. communication). It is tempting to speculate what the targets for metal ions in infected cells might be. In the case of rhinovirus, the proteases have been shown to be inhibited by zinc ions (Cordingley *et al.*, 1989). Similar results were obtained for the 3C polymerase *in vitro* (Hung *et al.*, 2002). However, as the metal ion balance in a eukaryotic cell is finely tuned, it is of major importance to analyze effects of metal ions on proteases in a cellular context.

A highly interesting property of these compounds is the fact that they can inhibit both + strand RNA viruses such as HRV and - strand RNA viruses as influenza. Although the mechanistic basis for inhibition might be different in both viruses, it is an interesting question whether these substances can trigger selected pathways of an unspecific antiviral response comparable to the induction of the “antiviral state” by interferon. Elucidation of these pathways would greatly deepen our understanding of virus-host interactions and facilitate therapeutic interventions by chemical molecules.

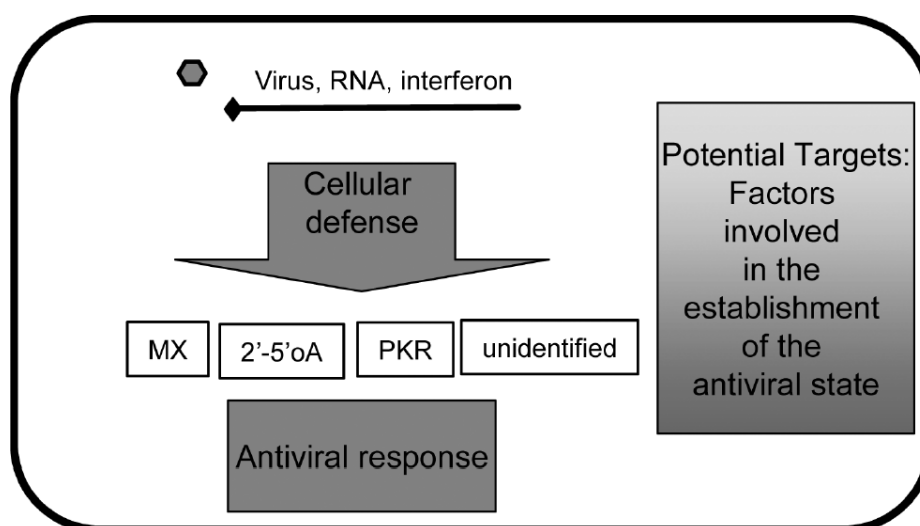


Figure 3. Induction of components of the antiviral defence system as an antiviral strategy

## 4.2 siRNA

As conventional antiviral strategies face several problems such as ineffectivity, toxicity and resistance, new approaches were taken also in the field of respiratory viruses.

RNA interference (RNAi) or RNA silencing are common designations of specific posttranscriptional gene silencing (PTGS). Small interfering RNAs (siRNAs) down-regulate gene expression by binding to complementary messenger RNAs and either triggering mRNA elimination or arresting mRNA translation into protein (miRNA) (Plasterk 2002; Carrington *et al.*, 2003; Denli *et al.*, 2003; Matzke *et al.*, 2003). This powerful technology has been widely employed to manipulate gene expression in diverse hosts and to identify gene function. RNAi is heavily used in basic research, and RNAi-based drugs are also being developed against human diseases, tumor and metabolic disorders. Consequently, this technique is now employed to inactivate viral genes and thus block viral replication (Carmichael 2002).

siRNA molecules mediating posttranscriptional gene silencing were originally discovered in plants and *caenorhabditis elegans*. In plants, there is evidence that RNAi has a role in the defense against viruses, as arabidopsis strains defective in posttranscriptional gene silencing are more susceptible to virus infections (Mourrain *et al.*, 2000). In addition, several plant viruses encode proteins that counteract RNAi-mediated silencing (Brigneti *et al.*, 1998; Voinnet 2001).

PTGS is mediated by siRNAs that are produced by type III endoribonuclease dicer. This enzyme digests large double-stranded RNAs into 21-23 nt double-strand RNA duplexes with 2 nt 3' overhangs. Importantly, these RNAs produced by dicer can be mimicked by synthetic RNAs (Elbashir *et al.*, 2001). In a second step, siRNAs are incorporated into a multicomponent nuclease complex termed the "RNA induced silencing complex" (RISC). The antisense-strand of the siRNA duplexes serves as a guide that directs RISC to the cognate RNA, which is subsequently degraded by RNAs. This process is highly efficient, as few RNAi molecules can trigger inactivation of continuously transcribed target genes over a prolonged period of time. Thus, siRNAs are usually more efficient than short antisense RNAs.

Guidelines for the choice of siRNAs are available. Preference should be given to siRNA target sequences not located in regions with heavy secondary structure such as the picornavirus IRES elements. siRNAs can be



produced (i) synthetically, (ii) by transcription from DNA templates, (iii) enzymatically by digestion of duplex RNA by cloned dicer or E. coli RNase III (Yang *et al.*, 2002). However, siRNAs have to be transported into the target cells. This is achieved by the use of such synthetic carriers as cationic lipids and polymers or with the help of viral vectors. The transient nature of the siRNA-mediated silencing is not believed to be a significant limitation when targeting diseases caused by rapidly replicating viruses. This is in contrast to a more problematic situation in chronic diseases and infections by such latent or integrating viruses as HIV.

#### 4.2.1 siRNA targeting antiviral genes

Attractive targets of siRNAs are plus-strand RNA viruses, as their genome is used both as an mRNA and as a template for replication. First experiments were carried out in poliovirus. For historic reasons, the latter is frequently used as a paradigm of picornaviruses. Based on their close relationship, results with poliovirus can be translated to human rhinovirus, albeit with caution. By targeting the capsid region and the 3D polymerase, virus titer was reduced by two orders of magnitude in a one step growth when cells were transfected before infection (Gitlin *et al.*, 2002). Virus inhibition occurred without need of interferon or such classical dsRNA-activated effectors as PKR and RNase L. This is in agreement with the view that RNA duplexes shorter than 30 bases do not activate the dsRNA-dependent protein kinase (Semizarov *et al.*, 2003).

However, poliovirus rapidly escapes highly effective siRNAs through unique point mutations within the targeted regions (Gitlin *et al.*, 2005). As picornaviruses exist as a quasispecies, it seems that pre-existing mutants can be selected rapidly. Combinations of siRNAs were more successful (Gitlin *et al.*, 2005). Another important animal virus of the picornavirus family, i.e. the foot and mouth disease virus (FMDV), could be inhibited by siRNAs against VP1 in cell culture and suckling mice (Chen *et al.*, 2004; Kahana *et al.*, 2004; Grubman *et al.*, 2005).

siRNAs have also been used to inhibit severe acute respiratory syndrome (SARS)-associated coronavirus replication. Several siRNAs were evaluated in cell culture against different viral genes (Zhang, R. *et al.*, 2003, 2004b; Qin *et al.*, 2004; Zhao *et al.*, 2005). siRNAs directed against spike sequences and the 3'-UTR can inhibit replication (Bitko *et al.*, 2005). siRNA targeting the leader sequence inhibited the replication of SARS-CoV more strongly than targeting the spike gene (Li *et al.*, 2005). Plasmid-mediated expression

of siRNAs targeting the RNA polymerase reduced virus titer, RNA and protein levels (Wang *et al.*, 2004).

The non-segmented genomic and antigenomic RNAs of RSV are a difficult target for siRNAs as they are tightly wrapped in the nucleocapsid protein N, which makes them inaccessible (Barik 2004). However, siRNAs targeting the NS1 gene (siNS1) were successfully used in mice treated intranasally with siNS1 nanoparticles. Decreased virus titers in the lung and decreased inflammation and airway reactivity could be observed. Thus, siNS1 nanoparticles may effectively inhibit RSV infection in humans (Zhang *et al.*, 2005). Also, intranasal delivery of siRNA against RSV P was successful in preventing infection in mice and in reducing the severity of the disease when added after infection (Bitko *et al.*, 2005).

One of the advantages is that siRNA approaches do not rely on the immune system. Essentially, all genes of influenza were targeted by siRNAs, except for hemagglutinin and neuraminidase (Ge *et al.*, 2003). Inhibition of virus multiplication could be obtained *in vitro*, in chicken embryos and in mice. For example, cationic carrier siRNA complexes were administered i.v. or intranasally or via DNA vectors from which siRNAs could be transcribed (Ge *et al.*, 2004a; 2004b). These siRNAs can prevent and treat influenza virus infection in mice. Other groups used siNP nucleocapsid or siPA components to protect mice from lethal challenge with a variety of influenza A viruses including potential pandemic H5 and H7 subtypes (Tompkins *et al.*, 2004). These data clearly show the potential of siRNAs as prophylactic and therapy for influenza virus infections.

#### **4.2.2 siRNA targeting cellular genes essential for virus multiplication**

An attractive idea is not to pursue the classical approaches of targeting the viral RNA but to target cellular genes which are identified as being essential for virus multiplication. So far, these strategies were not yet applied to classic respiratory viruses; however there are successful reports in related virus groups. Examples for such methods are targeting the translation factor La, the polypyrimidine-binding protein or eukaryotic initiation factor 2B gamma. As these factors are involved in the cap-independent translation of hepatitis C virus, siRNA targeting of these molecules blocks viral replication in cell culture (Zhang, J. *et al.*, 2004a). Similar results were obtained with picornaviruses (polio- and encephalomyocaeditisvirus) when cells were depleted of the polypyrimidine-tract-binding protein (Florez *et al.*, 2005). These studies demonstrate that viral infections can be combated by targeting

cellular co-factors required for replication. These approaches might lead to a more sustainable inhibition of viral replication as the emergence of resistant viruses is very unlikely.

The main limit of the siRNA technology is the sequence diversity of respiratory viruses. Resistant mutants were obtained in several viruses such as poliovirus. However, siRNA is a powerful technology that can be used to target viral genes or cellular co-factors and thus efficiently inhibit virus-induced pathogenesis.

## 5. CONCLUSIONS

Inhibiting respiratory viruses is a challenging task and many attempts have failed to show expected results. The diverse nature of the viruses involved is one reason for the problematic situation. Viruses have developed a number of strategies to evade common inhibition approaches. However, we believe that aiming at cellular rather than viral targets might improve the accessibility of viral diseases. It remains to be shown in further clinical trials whether new molecules are suitable for viral inhibition. New technologies such as siRNA techniques have yielded surprising results in the first years of development and offer a significant potential for treating viruses that have bothered people for many centuries.

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