The Human Rhinovirus: Human-Pathological Impact, Mechanisms of Antirhinoviral Agents, and Strategies for Their Discovery

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Abstract: As the major etiological agent of the common cold, human rhinoviruses (HRV) cause millions of lost working and school days annually. Moreover, clinical studies proved an association between harmless upper respiratory tract infections and more severe diseases e.g. sinusitis, asthma, and chronic obstructive pulmonary disease. Both the medicinal and socio-economic impact of HRV infections and the lack of antiviral drugs substantiate the need for intensive antiviral research. A common structural feature of the approximately 100 HRV serotypes is the icosahedrally shaped capsid formed by 60 identical copies of viral capsid proteins VP1-4. The capsid protects the single-stranded, positive sense RNA genome of about 7,400 bases in length. Both structural as well as nonstructural proteins produced during the viral life cycle have been identified as potential targets for blocking viral replication at the step of attachment, entry, uncoating, RNA and protein synthesis by synthetic or natural compounds. Moreover, interferon and phytoceuticals were shown to protect host cells. Most of the known inhibitors of HRV replication were discovered as a result of empirical or semi-empirical screening in cell culture. Structure–activity relationship studies are used for hit optimization and lead structure discovery. The increasing structural insight and molecular understanding of viral proteins on the one hand and the advent of innovative computer-assisted technologies on the other hand have facilitated a rationalized access for the discovery of small chemical entities with antirhinoviral (anti-HRV) activity. This review will (i) summarize existing structural knowledge about HRV, (ii) focus on mechanisms of anti-HRV agents from synthetic and natural origin, and (iii) demonstrate strategies for efficient lead structure discovery.

Key words: rhinovirus; antiviral; common cold; drug discovery
1. TAXONOMY AND HUMAN-PATHOLOGICAL IMPACT OF HUMAN RHINOVIRUSES

A. Classification of HRV and New Findings

Human rhinoviruses (HRV) are the major cause of upper respiratory tract symptoms, the so-called common colds in humans. Their name reflects the primary site of infection. Because HRV are nonenveloped, icosahedral viruses of small size with a diameter of about 30 nm (*pico* = small in Latin) that consist of an RNA genome, they were assigned to the family *picornaviridae*.

Currently, this virus family of the order *picornavirales* comprises the eight genera *enterovirus*, *hepatovirus*, *cardiovirus*, *kobuvirus*, *tesschovirus*, *erbovirus*, *aphthovirus*, and *par-echovirus* with 22 species and a multitude of serotypes. Because of high similarity in genome sequence and genome organization (Fig. 1), the former genera *rhinovirus* and *enterovirus* have been combined recently, keeping the existing name *enterovirus* (www.picornastudygroup.com/taxa/species/species.htm). An overview on the current taxonomy of picornaviruses pathogenic for humans as well as on newly proposed species of HRV is given in Table I. At present the genus *enterovirus* includes four approved human enterovirus (HEV) species (HEV-A, -B, -C, and -D) and two approved HRV species (HRV-A and -B) (www.picornastudygroup.com/taxa/species/species.htm). Since 2007, the global distribution of highly divergent HRV strains was reported. Based on the results of sequence, genomic, and phylogenetic analyses, it was proposed that these strains represent a new HRV species,
| Genus                  | Species                              | Serotypes                                                                 | Main clinical manifestations                                                                 |
|------------------------|--------------------------------------|----------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| Enterovirus            | Human enterovirus A                  | Coxackievirus A (CVA) 2–8, 10, 12, 14, 16, enterovirus (EV) 71, 76, 89–91 | Herpangina (CVA), meningitis, poliomyelitis, acute flaccid paralysis (AFP), gastroenteritis, exanthema, respiratory disease, hand-foot-and-mouth disease (CVA10, CVA16, EV71) |
|                        |                                      | CVA9, CVB1-6, echovirus 1–7, 9, 11–21, 24–27, 29–33, EV69, 73–75, 77–88, 97, 100, 101 | Meningitis, encephalitis, minor febrile illness, respiratory disease, pericarditis, myocarditis, diarrhea, AFP, hepatitis, acute hemorrhagic conjunctivitis (CVA24) |
|                        |                                      | CVA1, 11, 13, 17, 19–22, 24, EV96, poliovirus (PV) 1–3                      | Poliomyelitis (PV1-3), epidemic myalgia, Guillain-Barré syndrome, vomiting and diarrhea, AFP |
|                        |                                      | EV68 and 70                                                                | Pneumonia and bronchiolitis, acute hemorrhagic conjunctivitis (EV70), meningoencephalitis (EV70) |
|                        | Human rhinovirus A                   | Human rhinovirus (HRV) 1A, 1B, 2, 7, 9–13, 15, 16, 18–25, 28–34, 36, 38–41, 43, 44, 46, 47, 49–51, 53–68, 71, 73–78, 80–82, 85, 88–90, 94, 96, 98, 100, Hanks | Common cold                                                                                   |
|                        | Human rhinovirus B                   | HRV3-6, 14, 17, 26, 27, 35, 37, 42, 48, 52, 69, 70, 72, 79, 83, 84, 86, 91–93, 97, 99 | Common cold                                                                                   |
|                        | Human rhinovirus C<sup>a</sup>       | Have yet to be cultivated and/or assessed for immunological cross-reactivity | Acute lower respiratory tract infections                                                      |
|                        | Human rhinovirus D<sup>b</sup>       | HRV8<sup>8</sup>, 45<sup>8</sup>, 95<sup>8</sup>                           | Common cold                                                                                   |
|                        | Hepatovirus                          | Hepatitis A virus                                                          | Hepatitis                                                                                     |
|                        | Parechovirus                         | Human parechovirus 1 and 2 (previously classified as echovirus 22 and 23), 3–6 | Mild gastrointestinal or respiratory illness                                                   |
|                        | Kobuvirus                            | Aichi virus                                                                | Gastroenteritis                                                                                |

<sup>a</sup>Proposed as new species.<sup>2–4,6–7</sup>

<sup>b</sup>Proposed as new species.<sup>7</sup>

<sup>c</sup>Until now classified as serotypes of human rhinovirus A, proposed to belong to HRV-D.<sup>7</sup>
HRV-C. In 2009, a further proposal concerning a new potential HRV-D species was published after sequencing and analysis of all known HRV genomes. The approved and newly proposed species of the genus enterovirus share $\geq 70\%$ homology (average amino acid identity) in the precapsid protein P1 as well as in 2C and 3CD.

Different antigenic properties provide the basis for a further division of species into serotypes (Table I). About 100 rhinovirus serotypes are currently known. According to the currently approved taxonomy, most of them (75 and HRV Hanks) belong to HRV-A and 25 of them to HRV-B. The genome of all known HRV-A and -B serotypes as well as of several field isolates of HRV-A, -B, and -C has been sequenced completely. Viruses classified as HRV-C could not be grown in cell culture until now.

Phylogenetic analyses have been performed with partial sequences, as well as with the whole genome. The most recent and comprehensive analysis of all known HRV genomes revealed that (i) HRV-A and HRV-C share a common ancestor, which is a sister group to HRV-B, (ii) HRV-C represents a third species, and (iii) a basal divergence within HRV-A of three distinct strains that led to the proposal of a fourth species HRV-D.

**B. Association of HRV With Upper and Lower Respiratory Tract Infections**

HRV-A and -B most often induce a mild, usually self-limited upper respiratory illness in humans characterized by nasal stuffiness and discharge, sneezing, sore throat, and cough. The conventional term is common cold. The common cold is a heterogeneous group of diseases caused by numerous viruses that belong to several different families e.g. rhinoviruses, coronaviruses, enteroviruses, and adenoviruses. But, HRV represent the most common etiological agent worldwide. A large number of distinct strains circulate each year. Moreover, in a family or even in a single specimen, multiple HRV serotypes were detected simultaneously. By using RT-PCR and culture, it was shown that HRV induce 22–50% of upper respiratory tract infections in adults as well as children. Higher incidence has been described from September to November, and from April to May. In some years and perhaps some geographical areas, spring was a more important time for rhinovirus transmission. Although overall rates of respiratory illness are lower in summer, rhinoviruses are the most frequently isolated at this time of year. The incidence is inversely proportional to age. By age 2 years, 91% of the children have antibodies against rhinoviruses. In addition to common cold, HRV are also involved in acute otitis media in children. Moreover, data supporting a causative association with more severe lower respiratory tract infections of infants, elderly persons, and immunocompromised patients have been accumulated. Studies of childhood and adult asthma have shown that HRV infections can also trigger exacerbations in patients with asthma, chronic obstructive pulmonary disease, and cystic fibrosis. The recently discovered novel rhinovirus genotype HRV-C was associated with community outbreaks of influenza-like, acute upper respiratory infections and severe low respiratory tract infections of infants e.g. febrile wheeze, bronchiolytis, and asthma exacerbations, which peaked in fall and winter. In addition, the presence of HRV-C in the middle ear in patients with acute otitis media was demonstrated.

**C. Transmission of HRV**

HRV spread occurs by means of virus-contaminated respiratory secretions that contain a high virus concentration. Besides direct hand-to-hand transmission, small- and large-particle aerosol transmission of rhinoviruses has been shown. Children are important “vectors” for HRV transmission to family members. Moreover, studies with natural HRV-infected adults provided evidence that daily activities of infected people can lead to
contamination of environmental surfaces with HRV e.g. light switches, telephone dial buttons and handsets, and virus transfer to fingers of healthy individuals for infection.\textsuperscript{63,66} Because viral contamination of the hands plays an important role in transmission of HRV from person-to-person, interruption of this step of virus transmission presents a potential target for intervention. This was experimentally proved by treatment of hands by iodine\textsuperscript{67,68} or salicylic and pyroglutamic acid.\textsuperscript{69}

D. The Pathogenesis of HRV Infections

Observations from experimentally induced infections in normal adult volunteers helped to understand the pathogenesis of HRV infections.\textsuperscript{70–75} The 50% human infectious dose of rhinovirus is low and the infection rate between 70 and 80%. After the deposition of HRV on nasal or conjunctival mucosa, viruses are transported to the posterior nasopharynx by mucociliary action of epithelial cells. Specific receptors on epithelial cells in the adenoid area are used for binding and entry. Already 8–10 hr after intranasal inoculation, infectious virus can be detected.\textsuperscript{76} Virus shedding peaks on the second day after infection and decreases rapidly thereafter.\textsuperscript{75} But, small amounts of viruses were discovered in nasal secretions for up to 3 weeks after infection. Virus and/or viral RNA were demonstrated in the upper\textsuperscript{70} as well as lower respiratory tract.\textsuperscript{45,72,77,78} Using in situ hybridization, Arruda et al. (1995) detected viral RNA in a low number of ciliated cells in nasal biopsies. In the nasopharynx, a small portion of virus-positive ciliated as well as nonciliated cells was positive for viral RNA. In 2000 Papadopoulos et al. provided evidence that HRV may also lytically infect human bronchial epithelial cells in cell culture as well as in experimentally infected volunteers and induce the production of interleukin-6, -8, and -16. In agreement with these results, HRV RNA was detected in 24–45% of children and 10–18% of adults with pneumonia.\textsuperscript{79–82} Taken together, the results of natural cold studies as well as of experimental infection in human volunteers clearly demonstrate that HRV are able to replicate in the upper as well as in the lower airways.

HRV infection triggers vasodilation and increased vascular permeability in the nasal mucosa, leading to nasal obstruction and rhinorrhea. The mechanism is still incompletely understood because no histopathological changes were observed in nasal biopsy specimens from infected persons.\textsuperscript{75} This led to the suggestion that clinical symptoms are primarily caused by the inflammatory response of the host to the virus infection and not by the cytopathic effect (CPE) of HRV. Results of immunological investigations suggest a modest correlation between the concentrations of IL-6 and IL-8 in nasal secretions and the severity of symptoms in upper and lower HRV-induced respiratory tract disease.\textsuperscript{78,83} On day 2–4 after virus challenge, IL-6 and IL-8 concentrations were significantly greater in nasal secretions from experimentally infected symptomatic subjects than in those from infected asymptomatic or sham-challenged subjects. IL-8 has been proposed as a mediator of neutrophile infiltrations that are observed during symptomatic infections.

In experimental rhinovirus infection the onset of symptoms e.g. nasal stuffiness and discharge, sneezing, and cough was observed 10–12 hr after intranasal inoculation of the virus.\textsuperscript{76} In contrast to rhinovirus infections in adults, fever is found in 15% of children with upper respiratory tract infections.\textsuperscript{84} Other symptoms in children and adults may be hoarseness, headache, malaise, and lethargy. Sometimes viral infection is accompanied by bacterial complication, leading for instance to acute otitis media in about 20% of infected children, sinusitis, and pneumonia.\textsuperscript{79–81,85,86}

Experimental infection was also used to study the causation between rhinovirus infection and asthma as well as COPD exacerbations.\textsuperscript{78,87–93} It was shown that HRV infection enhances airway reactivity and predisposes allergic patients to develop late asthmatic
Rhino viral colds were associated with an increase in histamine responsiveness that was accompanied by a bronchial mucosal lymphocytic and eosinophilic infiltrate. In a recent study, an increased HRV-induced clinical illness severity in asthmatic compared with normal subjects was demonstrated. Strong relationships were shown between virus load, lower airway virus-induced inflammation, and asthma exacerbation severity. The results of this study also indicated that augmented Th2 or impaired Th1 or IL-10 immunity are likely important mechanisms. Mallia et al. provided evidence that low dose experimental rhinovirus infection in patients with COPD induces symptoms and lung function changes typical of an acute exacerbation of COPD. Viral replication and increased pro-inflammatory cytokine response were associated with symptomatic colds, increases in lower respiratory tract symptoms and reductions in forced expiratory volume in 1s or peak expiratory flow rate.

E. Requirements for Anti-HRV Agents

The epidemiological data and pathology of HRV infections explain their high medical and socio-economic impact. Millions of children and adults are taken ill with common cold every year, need medical consultations, are unable to attend school and go to work. Direct costs include hospitalization, medical fees, and symptomatic treatment. Moreover, exacerbations are the major cause of asthma and COPD morbidity, mortality, and health care costs associated with these diseases. To date, specific drugs that prevent or reduce rhinovirus infection are not available. Common cold can be treated only symptomatically with analgesics, decongestants, antihistamines, or antitussives and antibiotics are often wrongly prescribed. Because of the large number of circulating HRV serotypes, treatment with specific antiviral drugs is considered to be more striking than vaccination. Therefore, the search for new highly active synthetic and/or natural anti-HRV compounds is absolutely essential and represents an important area of antiviral research. Such an anti-HRV drug would have to be (i) with broad spectrum activity because of the high number of HRV serotypes, (ii) administered very early in infection to demonstrate a good antiviral effect because of the fast infection kinetics, (iii) very safe because of the broad application by millions of people, and (iv) directed against a highly conserved target with low risk of resistance development. Due to the very high error rates and the lack of proofreading ability in RNA polymerases of picornaviruses, naturally drug-resistant variants may exist in virus populations or resistant viruses can emerge under treatment. As with HIV, another highly variable RNA virus, the risk of resistance development and/or selection of resistant virus variants could be minimized by applying combination of drugs directed against different targets. Because clinical symptoms are suggested to be primarily caused by the inflammatory response of the host to the virus infection mediated by specific cytokines, a further advantage of drug combinations could be an additional immune-suppressive activity.

2. STRUCTURAL COMPONENTS, NONSTRUCTURAL PROTEINS, AND STAGES OF THE LIFE CYCLE OF HRV AS POTENTIAL TARGETS FOR SELECTIVE ANTIVIRAL DRUGS

The knowledge of structural components, nonstructural proteins that are necessary for viral multiplication, and stages of the viral life cycle is an essential precondition for the development of measures to prevent and treat HRV infection. The structure of HRV particles is well known. Infectious virions consist of an icosahedral protein shell (capsid) that surrounds and protects the genome, a single positive-stranded RNA molecule of approximately 7,400
The organization of the enterovirus genome is shown in Figure 1. The viral genomic RNA is infectious and encodes a single, long, open reading frame flanked by untranslated regions (UTR) at the 5' and 3' end. A small viral protein (VPg) is covalently linked to the 5' end. The 3' end is polyadenylated like cellular messenger RNAs. Structural components within these UTRs e.g. the cloverleaf and the internal ribosome entry site (IRES) of the 5'UTR play an important role in RNA replication as well as protein synthesis.98 The nucleotide sequence of some regions within these structures is highly conserved among enteroviruses. Their blockade could significantly inhibit viral replication.

The molecular structure of HRV-1A, HRV-2, HRV-3, HRV-14, and HRV-16 was determined by X-ray crystallography.99–105 The results show that the viral capsid is composed of 60 protomers of each of the three outer structural proteins VP1, VP2, and VP3 and of VP4 in the interior. A star-shaped plateau at the fivefold axis of symmetry, surrounded by a deep depression (canyon) and another smaller depression at the threefold axis were detected. Moreover, a hydrophobic pocket was found beneath the canyon floor. With exception of HRV-14 and HRV-3, this pocket is occupied by a fatty acid, the so-called pocket factor. These host cell molecules have been suggested to play an important role in the viral life cycle by providing transient stability to the capsid during its movement from one host cell to another.102 The outer surface of virions contains neutralization antigenic as well as host cell binding sites. The latter allow the virus to attach to molecules of the host cell membrane (adsorption), the receptors, and to start their life cycle.106–108 Based on their receptor use, two groups of HRV can be distinguished. The majority of HRV serotypes, the major group uses intercellular adhesion molecule-1 (ICAM-1) as their receptor.109 The 12 viruses belonging to the minor group attach to low density lipoprotein (LDL) receptor, very-LDL (VLDL) receptor, and LDLR-related protein on the cells whereat multiple receptors are involved.110–112 HRV of the major group apply the canyon as attachment site for binding to ICAM-1.113 In contrast, LDL receptors of minor group viruses bind near the tip of the five-fold vertex.114 HRV-87 has been shown to utilize a sialylated glycoprotein as a cellular receptor.115 Furthermore, a HRV-89 variant as well as wild-type HRV-54 can use heparan sulphate proteoglycans for cell attachment in addition to ICAM-1.116,117 The interaction of rhinoviruses with their receptors leads to virus concentration on the cell surface. It induces the release of the pocket factor and conformational changes in the capsid and mediates viral entry via endocytosis.118–121 Whereas ICAM-1 binding directly causes uncoating,122,123 release of the RNA genome from the capsid of LDL-bound minor group rhinoviruses is triggered by acidification of the endosomal, pH-dependent pathway.124–126 This detailed knowledge of the capsid structure and function as well as of the virus–receptor interaction offers a good possibility to develop antiviral drugs that interfere with the first steps of the viral life cycle, adsorption as well as uncoating.

After uncoating, rhinovirus proteins are synthesized by the translation of a single, open reading frame using cellular ribosomes. The resulting polyprotein of approximately 250 kD is cleaved by viral proteases 2Apro and 3Cpro into 11 final products (4 structural and 7 nonstructural proteins) immediately after translation.127,128 At first, both proteinases release themselves from the polyprotein by selfcleavage. The primary cleavage of the viral polyprotein between P1 and P2 is mediated by 2Apro. Thereafter, 3CDpro is released from the P3 precursor by autocatalytic cleavage. Next, 3Cpro and its precursor 3CDpro process proteins of the P1 (capsid proteins), P2, and P3 (nonstructural proteins) region. Interestingly, 2Apro cleaves also the eIF4GI/II component of the translation initiation factor eIF4F necessary for host cell protein synthesis,129–131 and 3Cpro and/or 3CDpro the RNA polymerase transcription factors TFIIID, TFIIIC, SL-1, and UBF.98 Therefore, effective inhibition of 2Apro and 3Cpro would not only inhibit virus replication but could also prevent the shutoff of cellular protein and RNA synthesis. Moreover, the active site of proteinases is highly conserved.
among enterovirus serotypes. This high conservation in conjunction with their important role in virus multiplication predestines these enzymes as targets for antiviral therapy.

The viral RNA polymerase 3D (3Dpol) represents another very important nonstructural protein of HRV. It forms a complex with both cellular and viral proteins, the RNA replication complex. This enzyme synthesizes viral minus-strand RNA and uses it as template strand for the synthesis of genomic viral RNA. VPg (3B) is the primer for negative-as well positive-strand RNA synthesis. Negative-strand, but not positive-strand RNA synthesis, is stimulated by 2Apro. Further viral accessory proteins include 2B, 2C, 2BC, and 3AB. Besides 3Dpol these proteins can play an important role in inhibition of viral RNA synthesis by antiviral compounds.

In summary, the knowledge of the structure of the viral capsid, proteases, and polymerase and their important function in the viral life cycle predestine these proteins as potential anti-HRV targets.

A. Experimental Approaches for Anti-HRV Studies

HRV grow in several human and some primate cells expressing the minor group LDL receptors and/or the major group receptor ICAM-1. Human cells susceptible to HRV infections include embryonic kidney, amnion, diploid fibroblasts from embryonic lung, tonsil, liver, intestine, and skin, adult fibroblast lines from aorta and gingival and the KB, HEP-2, and HeLa continuous cell lines. But, the susceptibility of HeLa cells and human fibroblasts to virus infection may vary. HRV multiplication also occurs in primary human airway fibroblasts and differentiated bronchial epithelium. The proportion of infectible epithelial cells was shown to be between 3 and 10%. But, enhanced levels of viral production were detected in poorly differentiated in comparison to differentiated epithelial cells. The degree of viral infection correlated with IL-6 and IL-8 induction in these cells.

Virus growth causes a typical CPE characterized by ballooning, refractiveness, granularity, and shrinkage of infected cells. The HRV-induced CPE, infectious virus titers, viral protein expression, and RNA synthesis can be chosen as parameters to evaluate the anti-HRV activity of compounds in cell-culture based assays. There are several methods for antiviral screening against HRV. The plaque reduction assay has been traditionally performed and accepted as the “gold standard” in antiviral testing. However, this test is laborious, time consuming, and the evaluation is subjective. Therefore, it is not suited for the routine antiviral testing. It was more and more replaced by methods based on quantification of protection from virus-induced CPE after drug treatment. So, the CPE in sample-treated and untreated cells has been compared by light microscopy. But this evaluation is also subjective. Another more objective approach is the spectrophotometric quantification of CPE results in neutral red or crystal violet uptake assays and the tetrazolium dye reduction method. It allows an excellent and rapid antiviral screening of large numbers of compounds using small amounts of extracts, natural, or synthetic compounds. Active samples can be scheduled for additional testing using other assays e.g. virus yield or plaque reduction assays, and for studies on the mechanism of action.

The activity of potential antiviral drugs has to be approved in vivo. Because of the high degree of species-specific variations in ICAM-1 preventing infection by major group HRV, practical animal models have been absent for a long time. Chimpanzees were infected with several HRV serotypes but without developing clinical signs. HRV do not induce infection in rabbits, guinea pigs, and weanling mice injected with HRV by different routes. One minor group HRV, serotype 2, was adapted to grow in mouse fibroblasts and used in a mouse model of rhinovirus infection in which growth could be demonstrated. Based on the fact that the LDL receptor family is highly conserved between human and mouse, Newcomb et al.
examined whether HRV-1B, another minor group virus, may infect mouse airways in 6- to 8-week-old female C57BL/6 mice. The authors demonstrated that this HRV serotype replicates and induces airway inflammation in vivo. These results strongly correspond to those of Bartlett et al. who established three novel mouse models of rhinovirus infection in BALB/c mice. In the first model, 6-week-old BALB/c mice were infected with HRV-1B. In the second model, transgenic BALB/c mice, expressing a mouse-human ICAM-1 chimera, were inoculated with the major group HRV-16. Rhinovirus-induced exacerbation of allergic airway inflammation is mimicked in the third model.

Due to the lack of a small-animal model for HRV infection until 2008, the experimental human challenge model has to be used to approve effects of potential antiviral drugs under controlled conditions in preclinical studies. Volunteers were experimentally inoculated with various serotypes e.g. HRV-4, HRV-9, HRV-23, HRV-29, and HRV-39 to examine the efficacy of potential antiviral drugs under standardized conditions. Examples and results of these studies with capsid-binders, protease, and RNA synthesis inhibitors, as well as interferons are described in the following sections.

3. SYNTHETIC INHIBITORS OF HRV REPLICATION

Antiviral agents that inhibit virus attachment, capsid uncoating, protein and RNA synthesis of picornaviruses are the best studied, and will be in the focus of this section.

A. Options to Prevent Virus Attachment and/or Uncoating

Inhibition of virus attachment and/or uncoating interrupts the viral life cycle at its beginning and prevents HRV infection. Options to prevent these early steps of the viral life cycle include (i) virus neutralization by HRV-specific antibodies, (ii) receptor blockade by antibodies directed against the cellular receptors ICAM-1 or LDL, (iii) by soluble receptor molecules, or (iv) by compounds interacting with the viral capsid.

Because of the high number of serotypes circulating often in parallel, application of HRV-specific antibodies is thought to be no promising approach for prevention or therapy of rhinovirus infection.

In contrast, antibodies directed against the cellular receptor or soluble receptor molecules of major or minor group HRV could inhibit 90 and 10% of HRV serotypes, respectively. Therefore, the strategy to prevent virus–receptor interaction by receptor antibodies or soluble receptor molecules has been extensively evaluated in vitro as well as in vivo. The antiviral activity of ICAM- and LDL-specific antibodies was confirmed in cell culture. Furthermore, the prophylactic effectiveness and safety of intranasally administered rhinovirus murine ICAM-1 antibody was assessed in two double-blind, placebo-controlled, randomized studies of volunteers experimentally inoculated with HRV-39. In the result, no toxicity related to antibody application was recognized. The higher dosage of 1 mg/subject of rhinovirus murine receptor antibody did not reduce overall infection or illness rates, but was associated with a 1–2 day delay in the onset of virus shedding and cold symptoms. Viral titers and nasal symptoms were significantly reduced on the second day after challenge. In summary, the monoclonal antibody to the cellular ICAM-1 was demonstrated to be not effective enough. A new strategy was the creation of multivalent Fab fusion proteins against ICAM-1. A new molecule, named CFY196 demonstrated a better avidity and in vitro potency against HRV over conventional MAbs. CFY196 is under development as nasal spray with the name of ColdSol.

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Antagonism of virus–receptor interaction was considered as another promising way to prevent HRV attachment to host cells. Soluble forms of fully or truncated ICAM-1,162–164 and LDL or VLDL-receptor concatemers165–167 exhibited antiviral activity against major and minor group HRV, respectively, in cell culture. Soluble forms of ICAM-1 compete with receptor binding sites on the virus capsid, hinder an early infection event such as entry or uncoating, or directly inactivate HRV due to the formation of empty capsids.163,168–170 A soluble LDL receptor fragment neutralized viral infectivity by aggregation.171 Concatemers of the third ligand binding module of the VLDL-receptor did not lead to viral aggregation but blocked the receptor binding sites and possibly inhibited viral uncoating by cross-linking the viral capsid subunits via multi-module binding.166 The antiviral activity of a truncated, soluble form of ICAM-1 was proved in HRV-16 infected chimpanzees.172 In randomized, double-blind, placebo-controlled trials, the safety and efficacy of intranasal administration of tremacamra, a soluble ICAM-1 in experimental HRV-39-induced colds in humans, was shown.173 No further development was reported for these agents.95

A further option to prevent virus attachment was described for low-molecular-weight compounds, the so-called capsid-binding agents, which enter the small hydrophobic pocket within viral capsid protein 1 beneath the ICAM-binding canyon of HRV.174,175 Zhang et al. showed that drug may integrate into mature viruses by diffusion as well as into progeny viruses during assembly.176 When HRV-14 and HRV-16 were grown in the presence of pleconaril, a higher occupancy occurred than when the drug was introduced into the already-assembled viruses. In doing so, capsid-binders induce conformational changes of the canyon of HRV-3 and HRV-14, hinder virus-receptor interactions, and prevent attachment to host cells.105,175,177–179 In addition, uncoating of both HRV serotypes was shown to be inhibited as a result of a potential loss of flexibility of the viral capsid after drug binding. In contrast to HRV-3 and HRV-14, capsid-binding compounds did not prevent attachment of HRV-1A. Results from X-ray studies showed that drug binding into the hydrophobic pocket of HRV-1A replaces the pocket factor but induces only very small conformational changes.180 Therefore, Kim et al. suggested that the observed conformational changes are too small to affect receptor binding. But, capsid-binding compounds prevented attachment of HRV-16 possessing a pocket factor like HRV-1A without distinct deformation of the pocket.102,176,181 Further results from comparative antiviral studies with different capsid-binding compounds and HRV, representative for the major and minor group, did not reveal a correlation between inhibition of adsorption and receptor grouping or antiviral grouping.182 The reasons for the difference in the mode of action of capsid-binding compounds related to attachment inhibition are not fully understood until now. Taken together, inhibition of RNA uncoating was found for all investigated serotypes after drug binding independent of receptor grouping whereas prevention of virus attachment was found to be an additional mode of action for individual viruses and/or drugs.

Till now, various potent compounds belonging to diverse chemical classes have been described as uncoating inhibitors. Just to give an impression of diversity, the structures of disoxaril and pleconaril,12,183–185 pirodavir and the oxime ether,14,141,142,186,187 the isoxazole derivate compound,19,143,188 the imidazole derivative SCH 38057,189–191 the chalcone Ro 09-0881,192,193 4′,6 dichloroflavan and isoiflavan,137,194 the pyridine derivative MDL 20,957,195,196 and the phenoxybenzene MDL-860197,198 that exhibit a potent anti-HRV activity (Table II) are shown as examples in Figure 2. They inhibit most of HRV serotypes and a couple of them also affect enteroviruses, however, with varying susceptibility. Based on variability of susceptibility to capsid-binders of different length, HRV serotypes were classified into two different groups, A and B.140 Several of the given examples of compounds were also clinically tested.
### Table II. Antiviral Target, 50% Inhibitory Concentration (IC₅₀) Against Rhinoviruses and Assays Used for the Determination of Antiviral Activity of Examples of Highly Active Synthetic Compounds Described in This Review (Chemical Structures are Presented in Figs. 2–4)

| Antiviral target | Synthetic compound | IC₅₀ (µM) | Assay | HRV tested | Citation |
|------------------|--------------------|----------|-------|------------|----------|
| Capsid binder    | Disoxaril          | 0.01–18.1| Plaque reduction assay | 35 serotypes | 139      |
|                  | Pleconaril         | 0.03–17.5; seven completely resistant HRV-B | Cell protection assay with crystal violet | All serotypes | 12       |
|                  | Pirodavir          | 0.003–39.0; four completely resistant HRV | Cell protection assay with MTT | All serotypes | 187      |
|                  | Oxime ether 14     | 0.002–0.02| Cell protection assay | 16 serotypes | 141      |
|                  | Compound 19        | 0.01    | Cell protection assay with crystal violet | HRV-2 | 188      |
|                  | SCH 38057          | 20.4–29.2| Plaque reduction assay | 6 serotypes | 191      |
|                  | Ro 09-0881         | 0.01–9.1| Plaque reduction assay | 12 serotypes | 193      |
|                  | BW863C             | 0.007   | Plaque reduction assay | HRV-1B | 194      |
|                  | Isoflavan          | ~0.48   | Plaque reduction assay | HRV-1B | 137      |
|                  | MDL 20,957         | 0.02    | Plaque reduction assay | 32 serotypes | 196      |
|                  | MDL-860            | 3.25a   | Virus yield reduction assay | 90 serotypes | 198      |
| 2A and 3C Proteinase | Homophthalimides | 4.2  | Virus yield reduction assay | HRV-2 and HRV-14 | 215 |
| 3C Proteinase    | Rupintrivir        | 0.003–0.08| Cell protection assay with XTT | 48 serotypes | 227      |
|                  | Compound 1         | 0.014–0.12| Cell protection assay with XTT | 35 serotypes | 231      |
| RNA synthesis    | 2-Furylmercury chloride | 0.02–2.5; 4 completely resistant HRV | Not described | 17 serotypes | 235      |
|                  | Ribavirin          | 73.8 and 123.0| Cell protection assay | HRV-2 and HRV-14, resp. | 239      |
|                  |                   | 102.5 and 159.8| Plaque reduction assay |           |         |
|                  | Enviroxime         | 0.17–1.0| Plaque reduction assay | 15 serotypes | 244      |
|                  |                   | 0.03–0.2| Cell protection assay with crystal violet | 12 serotypes | 245      |

*a*1 µg/mL reduced the virus yield of 72 serotypes by at least 1.0 log₁₀; 12 serotypes were intermediately inhibited, and 6 not.
Studying the development of clinically effective capsid-binders, the long road to the discovery of a clinically effective anti-HRV drug becomes apparent. One well-described example represents the discovery and optimization of capsid-binders from Sterling Winthrop Pharmaceutical group, the so-called WIN compounds. First inhibitors originated from juvenile hormone mimetics that demonstrated some activity against HRV-1A. Determination of the X-ray structure of HRV-14 helped to understand the compounds’ binding sites at the virus capsid. Results from subsequent X-ray studies of HRV-WIN compound complexes revealed the location and nature of binding sites and provided information concerning interactions within these sites. This knowledge was used for optimization and design of new compounds. Optimized WIN compounds, for example disoxaril and pleconaril (Fig. 2), consist of a methylisoxazol ring, a substituted phenoxy group, and a five-membered heteroatom ring and inhibit a broad spectrum of rhinoviruses and enteroviruses (Table II). In 1985, the first broad-spectrum WIN compound disoxaril (Win 51711)
was tested in clinical trials.\textsuperscript{139} The development of crystallurea in human volunteers treated with high doses as well as its low bioavailability (15\%) prohibited subsequent development. Thereafter, results from SAR and QSAR analysis were used to further enhance the potency and spectrum of activity. In 1992, another compound, WIN 54954, was clinically tested. It was not effective in humans infected with HRV-23 and HRV-29.\textsuperscript{153} Moreover, it was rapidly metabolized and induced a reversible hepatitis. Consequently, the further clinical development was stopped. The better understanding of pharmacokinetic properties of capsid binders and synthetic chemistry efforts led to the discovery of pleconaril, an orally bioavailable, well-tolerated capsid-binder that inhibits most rhinovirus as well as various enterovirus serotypes.\textsuperscript{12,183–185} In 2000, Schiff et al. published the efficacy of pleconaril in an experimentally induced coxsackievirus A infection in humans.\textsuperscript{200} In phase II placebo-controlled, natural cold trials, the drug produced a moderate reduction of 1–1.5 day in the medium time to elevation of illness compared with placebo.\textsuperscript{201} These results were confirmed in two subsequent pivotal studies.\textsuperscript{202} Besides the moderate clinical efficacy, these studies revealed that 13\% of baseline isolates were not susceptible to pleconaril and 11\% developed reduced susceptibility (defined as 10-fold increase in baseline value). In a subsequent study the relationship of pleconaril susceptibility and clinical outcomes in the treatment of common cold caused by rhinoviruses was demonstrated.\textsuperscript{203} Based on drug interaction, marginal treatment effect, and possibility of transmission of resistant viruses, the FDA did not approve the applied oral administration of pleconaril for the treatment of common cold. The molecular mechanism of drug interaction of orally given pleconaril was shown to be based on hepatic cytochrome P450 3A activation.\textsuperscript{204} To reduce adverse effects, Shering-Plough under license of ViroPharma completed a phase II clinical trial with an intranasal formulation of pleconaril for the potential treatment of common cold in high-risk populations in 2007. The results were not published until now.

Pyridazine analogues developed by Janssen Research Foundation represent another example for the long road to discovery of a clinically effective capsid-binder.\textsuperscript{186} In 1992, the broad-spectrum activity of pirodavir (Fig. 2; Table II) against rhinoviruses was published.\textsuperscript{187} In the same year the results of a randomized, double-blind, placebo-controlled trial to assess the therapeutic efficacy of intranasal pirodavir in natural common colds were described.\textsuperscript{205} Possibly as a result of poor water solubility and rapid hydrolysis of pirodavir, no clinical benefit was found. The problem of ester hydrolysis was resolved by the development of oxime ether analogues of pirodavir by Biota. An example is shown in Figure 2. Like pirodavir these new analogues are potent inhibitors of rhinoviruses.\textsuperscript{142} An advantage over pirodavir is their improved bioavailability. BTA-798, an antiviral analogue with long half-life and good oral bioavailability, was scheduled to a phase II clinical trial in 2008. The results have not yet been published. In summary, despite extensive research leading to the discovery of potent anti-HRV capsid-binders, no agent has been approved for prevention and/or therapy of rhinovirus-induced diseases so far.

B. Development of Protease Inhibitors

Nearly, the same conclusion has to be drawn for protease inhibitors. Because of their pivotal role for viral polyprotein processing and the high conservation of critical amino acids,\textsuperscript{132} 2A\textsuperscript{pro} as well as 3C\textsuperscript{pro} represent potential anti-HRV targets. Results from cell culture-based assays provided evidence that inhibition of HRV replication is in principle possible. For example, processing of the HRV-2 polyprotein was prevented by pyrrolidine dithiocarbamate treatment in virus-infected HeLa cells.\textsuperscript{206} In contrast to other enteroviruses,\textsuperscript{207–210} pretreatment of cell monolayers with different nitric oxide donors leading to S-nitrosylation of 2A\textsuperscript{pro} and 3C\textsuperscript{pro} had neither an effect on virus replication nor on HRV-induced IL-8 elaboration.\textsuperscript{211} The proteolytic activity of 2A\textsuperscript{pro} of HRV-14 was specifically inhibited by two elastase-specific
inhibitors, and an antiviral peptide representing a derivative of the caspase inhibitor zVAD.fmk. Homophthalimides, e.g. LY353349 (Fig. 3; Table II), were described as inhibitors of 2Apro as well as 3Cpro. In contrast to protease 3C, no structure–activity relationship studies have been reported for HRV 2A protease. Moreover, protease 2A accomplishes only one cleavage in HRV polyprotein, while protease 3C performs all other cleavages. After elucidation of the crystal structure of 3Cpro, computer modeling of structural features of protease inhibitors became possible. Furthermore, structure-based design was used to develop mechanism-based inhibitors of the 3C protease with potent antiviral activity against multiple HRV serotypes. Highly active compounds incorporate various Michael acceptor moieties, irreversibly bind to 3Cpro, and exhibit anti-HRV-14 activity in HeLa cells. Structure–activity studies were performed to optimize protease inhibitors. These efforts resulted in the identification of a highly active anti-HRV compound, AG7088 (rupintrivir; Fig. 3; Table II) that entered clinical trials. In cell culture, AG7088 inhibited a broad spectrum of laboratory HRV as well as clinical isolates. In a single-cycle, time-of-addition assay it demonstrated antiviral activity when added up to 6 hr after infection. Inhibition of HRV replication strongly correlated with reduction in the level of IL-6 and IL-8 release into cell supernatant, leading to the suggestion that this agent may not only block virus replication but also diminish symptoms. The pharmacokinetics and safety of rupintrivir were proved in two double-blind, randomized, placebo-controlled studies. Intranasal rupintrivir, administered as single doses of 4 and 8 mg or every 3 hr, six times per day, for 7 days, was safe and well tolerated. Three double blind, placebo-controlled clinical trials were conducted to assess rupintrivir nasal spray (2% solution) for prevention and treatment of experimentally induced rhinovirus colds in healthy volunteers. Rupintrivir prophylaxis reduced the proportion of subjects with positive viral culture by 26% and viral titers but did not decrease the frequency of colds. Drug treatment led to the reduction of the mean total daily symptom score by 33%. Subjects receiving rupintrivir also demonstrated significantly lower viral titers and RNA levels than placebo-treated subjects on days 2, 3, and 5 and on days 2 and 3, respectively. There was no influence on the proportion of subjects with positive viral culture and the frequency of colds. Clinical development was terminated because rupintrivir did not act in a subsequent natural infection study in patients. In parallel research efforts, an orally bioavailable inhibitor of

![Chemical structures of most active inhibitors of 2A and 3C protease of HRV.](image)
HRV 3C\textsuperscript{pro}, i.e. (E)-(S)-4-((S)-2-[(3-methyl-isoxazole-3-carbonyl)-amino]-2-oxo-2H-pyr- idin-1-yl)-pent-4-ynoylamino)-5-((S)-2-oxo-pyrrolidin-3-yl)-pent-2-enoic acid ethyl ester (Fig. 3; Table II), was discovered.\textsuperscript{225,231} Like rupintrivir, this compound is an irreversible inhibitor incorporating a Michael acceptor moiety that forms a covalent bond with the 3C protease active site cysteine. It demonstrated an antiviral activity against all HRV and related picornaviruses tested.\textsuperscript{231} In a phase 1 clinical study, compound 1 was shown to be safe and well tolerated. According to a publication of Patick, no further clinical development was planned for this compound.\textsuperscript{95}

C. Inhibition of Viral RNA Synthesis

The blocking of viral RNA synthesis during replication represents another site for chemotherapeutic interdiction. It was shown that, rhinoviral RNA can be targeted in a sequence-specific manner by deoxyribozymes,\textsuperscript{232} morpholino oligomers,\textsuperscript{233} and small interfering ribonucleic acids.\textsuperscript{234} The efficacy of the latter two approaches was confirmed in cell culture. In addition, 2-furylmercury chloride (Fig. 4; Table II),\textsuperscript{235} flavonoids for example 3-methylquercetin (Fig. 5; Table III),\textsuperscript{236} and pyrrolidine dithiocarbamate (Fig. 4) interfered with rhinoviral RNA synthesis and inhibited HRV replication in cell culture-based assays.\textsuperscript{206,237} The nucleoside analog ribavirin (Fig. 4; Table II) that inhibits a broad spectrum of RNA as well as DNA viruses acts also against HRV-2 in HeLa cells.\textsuperscript{238,239} The cellular inosine monophosphate dehydrogenase that controls de novo synthesis of purine nucleosides represents the principal target in the mode of action of ribavirin.\textsuperscript{240} Moreover, when ribavirin is incorporated into picornavirus RNA, it pairs equally well with either uracil or cytosine inducing mutations that can be lethal to RNA viruses.\textsuperscript{241} Further identified mechanisms of action for ribavirin include inhibition of genomic RNA capping, enhancement of host T-cell-mediated immunity against viral infections through helping to switch the host T-cell phenotype from type 2 to type 1.\textsuperscript{242} Another compound with potent anti-HRV activity in vitro is enviroxime (Fig. 4; Table II), a benzimidazole derivative.\textsuperscript{243,244} It inhibits viral plus strand RNA synthesis.\textsuperscript{245} In particular the 3A protein, which is involved in the initiation of plus strand RNA synthesis, was implicated as likely target of drug activity.\textsuperscript{246,247} However, results from another study suggest that enviroxime targets a complex of proteins and/or cellular factors and that the exact mechanism remains to be studied.\textsuperscript{248} Although there was a statistically significant reduction in clinical score in a prophylactic study with HRV-9-infected volunteers,\textsuperscript{152} enviroxime failed in experimentally induced HRV-4 and HRV-39 infection,\textsuperscript{149,151} and in clinical studies,\textsuperscript{248–250} because of poor bioavailability and side effects. In an attempt to overcome the marked hydrophobicity, water insolubility, and toxicity, Wyde et al.
incorporated enviroxime into liposomes and then tested the anti-HRV activity and toxicity of the liposome-incorporated enviroxime in cell culture. The liposome preparation of enviroxime inhibited HRV-1A and HRV13 as effective as the parent compound and was 10- to 50-fold less toxic. In contrast to free enviroxime, the liposome preparation was readily and

Figure 5. Chemical structures of natural anti-HRV compounds.

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### Table III. Reported Antiviral Activities of Natural Compounds Described in the Review (Chemical Structures Are Given in Fig. 5)

| Natural product                        | IC$_{50}$ (µM) | Assay/virus tested     | Positive control IC$_{50}$ (µM) | Proposed mechanism of action                  | Citation |
|----------------------------------------|----------------|------------------------|---------------------------------|-----------------------------------------------|----------|
| Farnesiferol B                         | 2.61           | Cell protection assay HRV-2 | Pleconaril 0.03                 | Capsid binder                                | 144      |
| Farnesiferol C                         | 2.51           | Cell protection assay HRV-2 | Pleconaril 0.03                 | Capsid binder                                | 144      |
| 6,7,8-Trimethoxy-coumarin              | 11.98          | Cell protection assay HRV-2 | Pleconaril 0.03                 | Capsid binder                                | 318      |
| Flavan                                 | 0.05           | Plaque reduction assay HRV-1B | –                             | Capsid binder                                | 149      |
| Arborinine                              | 3.19           | Cell protection assay HRV-2 | Pleconaril 0.03                 | Capsid binder                                | 318      |
| (++)-Thysanone                         | 47.1           | 3C protease assay HRV-14   | –                              | Inhibitor of 3C protease                     | 323      |
| 2-Methoxy-stypandrone                  | 4.6            | 3C protease assay HRV-14   | –                              | Inhibitor of 3C protease                     | 323      |
| 9,10-Phenanthra-quinone                | 1.4            | 3C protease assay HRV-14   | –                              | Inhibitor of 3C protease                     | 324      |
| Chrysosplenol C                        | 0.75           | Cell protection assay poliovirus type 1 | Guanidine HCl 310–1250 | Inhibitor of virus replication                | 317      |
| Quercetin 3-methylether                | 0.95$^a$       | Titer reduction assay poliovirus 1A/S3 | –                             | Inhibitor of virus                           | 320      |
|                                       | 0.03$^b$       | Virus yield reduction assay HRV-15 | –                             | Replication                                   | 236      |
| Kaempferol 3-methylether               | 0.67$^a$       | Titer reduction assay poliovirus 1A/S3 | –                             | Inhibitor of virus replication                | 320      |
| 4’,5-Dihydroxy-3,3’, 7-trimethoxyflavone| ~0.3           | Cell protection assay 20 HRV serotypes | –                             | Inhibitor of virus replication                | 192      |
| Glaucine                                | 22.0           | Cell protection assay HRV-14 | Disoxaril 1.5                   | Inhibitor of virus replication                | 327      |
| Oxoglucine                              | 0.3            | Cell protection assay HRV-14 | Disoxaril 1.5                   | Inhibitor of virus replication                | 327      |
| o-Coumaroylamide of 3 aminomethylglaucine | 15.0           | Cell protection assay HRV-14 | Disoxaril 1.5                   | Inhibitor of virus replication                | 327      |
| p-Coumaroylamide of 3 aminomethylglaucine | 13.0           | Cell protection assay HRV-14 | Disoxaril 1.5                   | Inhibitor of virus replication                | 327      |
| Axillarin                               | 1.82           | Cell protection assay HRV-2 | –                              | Inhibitor of virus replication                | 319      |
| Raoulic acid                            | <0.27          | Cell protection assay HRV-2 | Ribavirin 356.5 (HRV-2)         | –                                              | 325      |
|                                       | 0.51           | Cell protection assay HRV-3 | –                              | –                                              |          |

$^a$99% effective dose.
$^b$Maximum tested concentration.
successfully delivered by small-particle aerosol to the upper and lower respiratory tract of mice. In another attempt to overcome the disadvantages of enviroxime, several benzimidazole as well as nonbenzimidazole analogs were synthesized and studied.\textsuperscript{252–255} Even though some compounds were better bioavailable and could be administered orally,\textsuperscript{254} none of these compounds was tested in clinical studies.

4. APPLICATION OF INTERFERONS

Besides virus-specific targets, cellular inhibitors like interferons may represent a therapeutical approach. Among other activities, interferons exhibit antiviral activity. The advantages of interferon application include the broad spectrum of activity and low risk of resistance development. Human leukocyte and fibroblast as well as recombinant human $\alpha$ interferons prevent the HRV-induced CPE in cell culture whereas a variation in sensitivity was observed.\textsuperscript{256–258} Intranasally applied recombinant interferon $\alpha$ and interferon $\beta$ have been shown to be effective in humans when provided prophylactically both in experimental and natural rhinovirus colds.\textsuperscript{259–264} Significant reductions in illness frequency, mean symptom score, nasal secretion weights, and frequency of virus isolation were observed. In contrast, recombinant interferon $\gamma$ did not prevent HRV infection or illness and may enhance the symptoms.\textsuperscript{265} Little to no therapeutic effect was found in patients with common cold after interferon treatment.\textsuperscript{150,266} Moreover, blood-tinged mucus and nasal bleeding were described as side effects.\textsuperscript{263,267} Combining interferons with dichloroflavan, enviroxime, chalcone Ro-09-0410 produced synergistic increases in antiviral activity in vitro against HRV-2 and HRV-9.\textsuperscript{268} An attempt to demonstrate synergy between the anti-HRV effect of recombinant human rHuIFN $\alpha$ and enviroxime in HRV-9 and HRV-14-infected volunteers failed.\textsuperscript{269} According to the authors, the main reason for this failure may be the rapid removal of enviroxime from the nose when given intranasally.

5. ANTI-HRV AGENTS FROM NATURE AND PROPOSED MECHANISM OF ACTION

A. Impact of Natural Products

Nature provides an astonishing pool of secondary metabolites biosynthesized from living organisms such as plants, fungi, protozoan, insects, and other animal sources. In contrast to synthetic compounds, natural products are characterized by an overwhelming chemical diversity. Previously, the chemical diversity space between these two groups was evaluated with respect to drug substances by Feher and Schmidt.\textsuperscript{270} It is shown that combinatorial compounds densely populate a small area, whereas natural products cover a wider range quite similar to the chemical space occupied by drug substances. The authors accordingly suggest that combinatorial libraries that mimic the distribution properties of natural products might be more biologically relevant. One may assume that secondary metabolites evolved as reaction to their target receptors related to defence, protection, attraction, and signalling. These adaptation processes have enriched not only the metabolites’ structural diversity but have also optimized drug-like metabolic traits likely to have favorable pharmacokinetic properties.\textsuperscript{271,272} It is this evolutionary concept that gives the pool of natural products the greatest source of scaffold diversity with molecules of biological relevance.

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Newman and Cragg analyzed the number of drugs approved between 1981 and 2006 and circumstantiated that especially the anti-infective area is strongly dependent on natural products and structures derived from natural scaffolds. The anti-infectives including the antiviral vaccines are with 22.8% or 230 launched drugs by far the major category with only about 30% being synthetic in origin. From 1981 to 2006, 78 vaccines and antiviral drugs have been approved. Excluding the high number of vaccines (25) and biologicals (12), most of the 41 small antiviral molecules are based on nucleoside structures or on peptidomimetics; only 16.7% are classified as totally synthetic drugs. However, till now, neither a synthetic nor a naturally derived anti-HRV drug substance has been approved for the treatment or prevention of HRV infections.

Intensive research and development efforts in the field of natural products revealed several inhibitors of viral attachment and entry, and inhibitors of viral protease from natural sources. The efficacy of natural products is not only reflected by statistics of launched drugs but also by empirical knowledge gained over centuries by successful application of natural-based ethnomedicinal products such as plants, culinary herbs, and spices. Phytochemical and pharmacological work performed with ethnomedicinal anti-HRVs mainly from plants revealed a high number of active metabolites from different chemical classes, e.g. coumarins, flavonoids, alkaloids, quinones, terpenoids, polyphenols, and polysaccharides.

Natural products include complex extracts and their chemical entities, which are biosynthesized by nature. For an unambiguous presentation of anti-HRV natural products it is of prior importance to first distinguish between a single chemical entity from nature, i.e. an isolated, purified natural compound, on one hand, and a natural preparation comprising hundreds to thousands of constituents, mainly secondary metabolites, on the other hand. If natural preparations are derived from plants, they might also be labelled as botanicals, phytoceuticals, or phytotherapeutic agents. These multicomponent preparations might show a varying profile of their constituents depending on the used species, origin, collection time, plant parts, extraction procedures, preparation methods, and manufacturing processes, just to mention a few important elements. These parameters affect the final product in terms of the qualitative and quantitative composition of chemical constituents, which may have an impact in biological activity. Accordingly, studies performed with phytochemically not specified extracts or nonstandardized preparations often suffer from irreproducible and incomparable results.

**B. Anti-HRV Natural Preparations**

A wide variety of natural preparations showed to be acting therapeutically in HRV and other viral infections with often complementary and overlapping antiviral mechanisms of action. Most of these remedies are described in ethnopharmacological sources or handed down for generations. They usually consist of simply prepared natural items whose chemical composition is complex. Many of the contained secondary metabolites, possibly active principles, have never been examined chemically or biochemically using modern medical knowledge. They are however components of plant medicines, which have stood the test of time and as such may offer clues of great interest to medicinal chemists. A clear advantage of the application of these products is their absent or relatively low toxicity due to a usually long-term empirical trial.

Although the knowledge of the immuno-pathogenesis of RV-induced diseases remains limited, the host defense function of the airway epithelium plays an important role in the innate-immune response to HRV-infection. Host cells respond by the production of mediators with antiviral activity such as type I interferons and nitric oxide, and produce cytokines and chemokines that influence the subsequent induced innate- and specific-immune
response. These processes are beneficial in facilitating clearance of virus from the respiratory tract, but also cause immuno-pathology. Following HRV-infection, disease severity is dependent on direct, harmful effects of the virus as well as tissue damage as a result of the host antiviral immune response. Accordingly, a number of agents with phenomenological effects against common cold have shown to exert their activity more in the field of regenerating tissue damage than on a direct anti-HRV effect.

Several herbal remedies consisting of a multitude of secondary metabolites from different chemical classes may attribute in a beneficial way for the treatment of common cold by reducing symptom severity and duration due to their immune-modulating, anti-oxidative, and anti-inflammatory properties. Beside these commonly observed bioactivities of natural products, multicomponent mixtures like botanicals often show overlapping symptomatic effects as well as synergistic and/or additive properties. Thus, it is a challenging endeavor to track down an observed phenomenological effect of a complex mixture on a molecular level.

The following section explores the significance and current knowledge of selected botanicals for the prevention and therapy of common cold. Questions about (1) clinical evidence of efficacy, (2) the constituents or at least the chemical classes that are involved in the observed anti-HRV effect, and (3) the involved pharmacological targets were covered as far as possible.

1. **Echinacea** (*E. angustifolia*, *E. purpurea*, *E. pallida*)

Echinacea preparations include expressed juice from aerial parts as well as extracts of roots or aerial parts, or both, from one or more species of the genus Echinacea (*E. angustifolia*, *E. purpurea*, and *E. pallida*). They are the most recognized botanicals for prevention and treatment of common cold and flu, and account for the second top-selling herbal products in the US-market. Accord-ingly, Echinacea has come under much scientific scrutiny. The high number of studies dealing with the effectiveness of Echinacea for preventing and treating the common cold from clinical trials was recently reviewed by Woelkart et al. The authors summarized the findings of the meta-analyses regarding the 16 randomized controlled trials evaluated in the Cochrane database, the 14 randomized clinical trials analyzed by Shah et al., and the experimental HRV-infection studies pooled by Schoop et al. To sum up, the clinical data on Echinacea so far are not fully consistent, mainly based on problems inherent in assessing the efficacy of Echinacea preparations, such as lack of comparability of available preparations, study design, and outcome. Nevertheless, the meta-analyses showed some evidence that preparations based on the aerial parts of *Echinacea purpurea* might be effective for the early treatment of colds in adults. Echinacea showed to decrease the odds of developing the common cold by 58% and the duration of a cold by 1.4 days. Similarly, the evaluation of three induced rhinovirus prevention studies revealed the odds of experiencing a clinical cold were 55% higher with placebo than with Echinacea.

Stepping into a molecular level, several constituents found in Echinacea species could potentially affect the symptoms of common cold. Chemically identified substances include polysaccharides and glycoproteins, caffeic acid derivatives (especially cichoric acid and echinacoside), and lipophilic polyacetylenes and alkamides. Pharmacological studies have shown that cichoric acid, alkamides, glycoproteins, and polysaccharides possess immunomodulatory activity. Additionally, alkamides have been reported to exert not only anti-inflammatory effects but also cannabinomimetic properties, which are suggested as molecular mode of action of Echinacea alkamides as immunomodulatory agents. Raduner et al. showed that some Echinacea alkamides exert cannabinoid type 2 receptor-dependent and independent immunomodulatory effects on cytokine expression. Different Echinacea constituents were evaluated for their anti-oxidative effects measuring the inhibition of in vitro Cu(II)-catalyzed oxidation of human low-density lipoprotein. Thereby, the major
caffeic acid derivatives, cichoric acid and echinacoside, showed the highest anti-oxidative effects, which was even higher when combined with a natural mixture of alkamides.\textsuperscript{285} Sharma et al. used cytokine antibody arrays to investigate the changes in the pro-inflammatory cytokines and chemokines released from human bronchial epithelial cells exposed to HRV 14.\textsuperscript{286} Application of two chemically characterized Echinacea extracts showed a reversion of the stimulated release of numerous pro-inflammatory cytokine-related molecules, e.g. for the cytokine IL-6, and the chemokines IL-8 and eotaxin. In a similar study, an Echinacea extract rich in polysaccharides and another rich in alkamides and caffeic acid derivatives were as well able to neutralize the effects of HRV-infected epithelial cells.\textsuperscript{287} Using gene expression analysis both studies revealed the anti-HRV benefit of Echinacea preparations being involved in multiple immune response signaling pathways. Taken together, the numerous pharmacological findings from literature, the potential of Echinacea preparations, and their constituents to combat or prevent common cold can be deduced to immune modulating, anti-inflammatory, and anti-oxidative properties that may also act in some combination of these event, rather than acting directly on HRV.

2. \textit{Garlic (Allium sativum)}

Garlic cloves have been used traditionally to treat a number of infectious diseases. However, only few confirmatory studies have been published regarding the traditional antiviral uses. The clinical effectiveness of garlic on the prevention of common cold was investigated by Josling in 2005, who published a double-blind, placebo controlled study assessing 146 patients more than a 12-week treatment period with an allicin-containing garlic supplement.\textsuperscript{288} Common cold infections and symptoms were recorded in a daily diary. Patients in the treatment group had significantly fewer colds than patients in the placebo group (24 vs. 65, $P < 0.001$) who also had a longer duration of symptoms (5.01 vs. 1.52 days, $P < 0.001$).

As soon as the garlic is chewed, cut, or pressed, its main ingredient, the sulphur containing alliin, is broken down by the enzyme alliinase to the thiosulfinate allicin. By steam distillation allicin is transformed to diallyl disulfide and diallyl trisulfide that are responsible for the distinctive smell of garlic. Further, allicin transformation compounds, such as E- and Z-ajoene, are not found in fresh garlic, but in lipophilic extracts. By investigation of different garlic extracts and isolates against a number of different human pathological viruses, Weber et al. could show that allicin was the most active virucidal component from fresh garlic and fresh extracts.\textsuperscript{289} Results of the direct pre-HRV-2-infection incubation assay let suggest allicin to bind to the viral protein capsid, leading to a subsequent inhibition of viral adsorption and penetration. Although the garlic thiosulfimates are endowed with significant cytotoxicity, the antiviral effects were obtained in nontoxic concentrations.\textsuperscript{289} Beside the direct anti-HRV effect of fresh garlic extract and allicin, a number of human immune functions were found to be enhanced in vitro by aqueous garlic extract, its polar, and thiosulfinate fractions.\textsuperscript{290}

3. \textit{North American Ginseng (Panax quinquefolium)}

In North America, \textit{Panax quinquefolium}, the ginseng species indigenous to both Canada and the United States, has been a popular herbal remedy to combat stress, and to modulate both natural and acquired immune responses. American ginseng root extracts, rich in poly-furanosyl-pyranosyl-saccharides, have been found efficacious in the prevention of upper respiratory infections in immunocompetent healthy adults.\textsuperscript{291,292}

In a randomized, double-blind, placebo controlled trial, 200 mg of a proprietary American ginseng root extract was given to 43 community-dwelling elderly adults (age $> 65$ year) twice a day more than a “cold and flu” season period of 4 months. One month into the study, all participants received an influenza vaccination. During the first two months, no significant differences in duration and incidence were observed when compared to placebo. However, during the last two months significantly fewer subjects of the ginseng group
reported acute respiratory syndromes than the placebo group (32 vs. 62%). Additionally, the duration of respiratory symptoms was reduced by 55% in the ginseng group.291

In a similarly arranged trial, 323 healthy adults (ages 18–65 years) with a history of at least two colds the previous year commenced a 4 month study at the beginning of a cold and flu season.292 They received two 200 mg capsules daily of standardized American ginseng root extract or a placebo. Outcomes measured were number of colds including symptom severity and total number of symptomatic days. A therapeutic effect was reported regarding symptom severity and fewer symptom days that were 31 and 34.5% lower in the ginseng group than in the placebo group.

A phase II randomized, controlled trial of 2 dosing schedules of American ginseng root extracts, rich in poly-furanosyl-pyranosyl-saccharides, evaluated the safety, tolerability, and efficacy in a pediatric population already suffering from an upper respiratory tract infection. The results showed no serious adverse events and a good tolerability of both ginseng doses; however, frequency and severity of symptoms were not significantly different among each of the three treatment groups, i.e. standard dose, low dose, and placebo.293

The most prominent constituents of the genus *Panax* are the triterpene saponins ginsenosides. They are known to have numerous pharmacological activities such as anti-cancer, anti-diabetes, antiviral, and anti-atherosclerosis effects. Some compounds of this chemical class showed to be responsible for the immunostimulant activity of ginseng.294 On the other hand, the efficacy of a polysaccharide-rich extract of American ginseng was compared with an extract rich in ginsenosides on systemic and gut-associated immune function. The authors of this study investigated the lymphocytes isolated from spleen, mesenteric lymph nodes and Peyer’s patches, and immune cell proportions and cytokine production from Sprague–Dawley rats. They could show that the polysaccharide-rich ginseng extract modifies the rats’ systemic immune responses and affects the gut-associated immunity in a manner distinct from that of the ginsenoside-containing extract of American ginseng.295

A direct antiviral activity of ginseng constituents could be attested for the polysaccharides on rotavirus infection in MA104 cells. The triterpene saponins, however, did not exhibit any rotavirus infection-inhibitory activity in this study.296 A moderate in vitro virucidal effect (ID$_{50}$ 62 µM) of the ginseng saponin chikusetsusaponin III against herpes simplex virus type I was detected by Fukushima et al. This compound exhibited an intracellular inhibitory activity, but could only marginally affect the viral proteins postinfection.297

**4. Bu-zhong-yi-qi-tang/Hochu-ekki-to**

The ancient Chinese formula Bu-zhong-yi-qi-tang (Japanese name Hochu-ekki-to) is a traditional herbal medicine in China and Japan that is composed of ten species of medicinal plants, namely *Astragali radix*, *Atractylodis lanceae rhizoma*, *Ginseng radix*, *Angelicae radix*, *Bupleuri radix*, *Zizyphi fructus*, *Aurantii nobilis pericarpium*, *Glycyrrhizae radix*, *Cimicifugae rhizoma*, and *Zingiberis rhizoma*. This formula is reported to have various immunomodulatory,298–300 and anti-inflammatory activities.301 Yamaya et al. recently investigated the effects of Hochu-ekki-to in cultures of human airway epithelial cells infected with HRV-14.302 The output of virus, associated levels of viral RNA, and the production of ICAM-1, cytokines and acidic endosomes in cells were measured. In airway epithelial cells Hochu-ekki-to was able to decrease virus output and susceptibility to HRV infection by decreasing ICAM-1 and by blocking the entry of viral RNA into the cytoplasm from the endosomes. Glycyrrhizin, a major component of one herbal ingredient of Hochu-ekki-to, i.e. *Glycyrrhiza glabra*, was able to reduce supernatant virus titers dose-dependently, with a maximum effect between 0.12 and 0.6 µM. However, no clinical trials with representative numbers of subjects are published so far.

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5. **Umckaloabo (Pelargonium sidoides)**

*P. sidoides* and *P. reniforme* form the origin of the popular drug Umckaloabo. This herbal remedy from South Africa has found entrance in Western medicine mainly as aqueous ethanolic root extract from *P. sidoides* for the treatment of infections of the respiratory tract. The efficacy of Umckaloabo compared with placebo has been evaluated in 103 adults suffering from common cold by Lizogub et al. The applied herbal preparation was well tolerated by the patients. The study demonstrated only a weak efficacy of Umckaloabo compared to placebo after 5 days. After 10 days, however, the *P. sidoides* extract significantly reduced the severity of symptoms and shortened the duration of the common cold compared with placebo. Just recently, Timmer et al. selected randomized controlled trials examining the efficacy of *P. sidoides* preparations for the treatment of various acute respiratory infections and analyzed their efficacy and safety. The authors concluded that Umckaloabo may be effective in alleviating symptoms of acute rhino-sinusitis and the common cold in adults. It may be effective in relieving symptoms in acute bronchitis in adults and children, and sinusitis in adults. Reliable data on the treatment for other acute respiratory infections however were not obtained.

Identification of the metabolites from Umckaloabo revealed a high number of different chemical classes, such as phenolic and cinnamic acids, tannins, flavonoids, and coumarins. Antibacterial activities of Umckaloabo against different pathogens have been reported. Phenols, coumarins, and tannins have been identified to contribute with moderate antibacterial activities, however, cannot explain the effect of the whole extract (reviewed by Kolodziej). Additionally, *P. sidoides* extracts have been reported to significantly activate the nonspecific immune system by induction of TNF and NO-release, and IFN-like activities. These effects are assumed to contribute to the controversially discussed potential of *P. sidoides* extract for the treatment of upper respiratory tract infections. Only one study reports a direct antiviral effect, i.e. a clear dose-dependent anti-herpes simplex virus activity for the aqueous root extract of *P. sidoides*. Further pharmacological studies are needed to elucidate potential direct anti-HRV properties of Umckaloaba and its constituents.

6. **Carrageenan (Sulphated Polysaccharides)**

Carrageenan, a mixture of different polysaccharides, which is mainly extracted from red seaweeds, has been extensively used in food, cosmetic and pharmaceutical industry as a thickener and gelling agent. It has previously shown an antiviral efficacy against several viruses. In a recently published study, Lambda-, Kappa, and Iota-carrageenan were investigated for their anti-HRV inhibiting potential. At a concentration of 200 μg/mL Iota-carrageenan, a sulphated polysaccharide, was able to fully inhibit virus-induced cell death in HRV-2 infected HeLa cells. Based on their studies, Grassauer et al. concluded that Iota-carrageenan is effective against different HRV-serotypes on primary human epithelial cells. It is hypothesized by the authors that Iota-carrageenan might create a hostile environment for HRV and thereby block viral entry and replication. Because of its safe application and proved in vitro efficacy, Iota-carrageenan deserves consideration as a candidate for clinical trials for prevention and therapy of HRV-induced common cold.

7. **Difficulties for the Development of Botanicals Combating Common Cold**

The level of knowledge on the impact of the six botanicals on HRV-infection discussed above is different and heterogeneous. The best studied herbal remedy associated with common cold, i.e. Echinacea, showcases the innate problem connected with multi-component mixtures: starting from the late nineties till June 2009 some 100 original articles have been published to this topic and tried to elucidate questions concerning efficacy, molecular mechanism, and bioactive ingredients of Echinacea. Although some evidence is provided for the effects of extracts, chemical classes as well as well-defined constituents on specific targets and

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pathways, the findings cannot be deduced to a common denominator. Further, results from clinical trials often suffer from lack of comparability, because of using different study designs, outcome measures, and overall the application of different preparations.\textsuperscript{279} A proper quality analysis and characterization of the preparation under investigation is mandatory and should follow the recommendations and guidelines for reporting clinical trials for herbal medicine.\textsuperscript{311} As underlined before (Sections 5.1. and 5.2.), the chemical complexity of a natural preparation might be beneficial in terms of synergistic, additive, and overlapping effects caused by the multitude of evolutionary trimmed metabolites, which may attribute with modulating multi-target effects. On the other side, exactly this fact is hardly compatible with the proper assignment of an activity to a defined chemical entity according to Western medical practise. In contrast to a single compound (synthetic or naturally based), the chemistry of a botanical is not only complex but also varying. The analytical profile and in term the pharmacological profile of the investigated samples can differ substantially. Accordingly, the quantitative and qualitative comparison of different studies resulting from botanicals is by far more complex than those performed with pure single compounds, and may also explain why so little emphasis from pharmaceutical industry has been put into the further development of even promising natural preparations.

6. STRATEGIES FOR THE DISCOVERY OF ANTI-HRV LEAD STRUCTURES

In general, the search for potent, selective, nontoxic compounds that might be developed further to a drug substance is a multidisciplinary, time- and cost-consuming process. Therefore, strategies for a target-oriented discovery of lead-structures either from nature or synthesis are in high demand. Some of them will be discussed in the following section, providing examples from anti-HRV research.

A. Ethnopharmacological Approach

As already mentioned, nature provides an extremely rich pool of bioactive natural products. It is however a challenging endeavor to find exactly those compounds that show an activity on the focused target. In natural product research hints from folk medicine are a valuable starting point to dig for lead structures of certain interest. The majority of active principles from higher plants has been discovered as a result of ethnopharmacologically directed pharmacognostic research.\textsuperscript{312,313} Oral or written indications for a beneficial application of a natural material first need a critical evaluation as to the selection of the correct material, its medicinal preparation, the kind of application, and overall the pharmacological profile. A rational criticism of often anecdotal efficacy from traditional medicine is a mandatory attitude to avoid overinterpretation of handed-down information.\textsuperscript{314} In case of pretended anti-HRV remedies, the reported biological efficacy obviously suffers from being restricted to respiratory diseases, which might be caused by a panel of bacterial or viral infections. Thus, an approved remedy may affect the microbes, or show an immune modulating or even anti-inflammatory effect, or a combination of these. The holistic access is an innate character of ethnopharmacology and needs to be tracked down to the specifically involved target/s.

In a recently published study focusing on the discovery of HRV-capsid binders from nature using a pharmacophore-based virtual screening of an ethnopharmacologically biased 3D-multiconformational database, some 30 secondary metabolites were predicted to act as capsid-binding inhibitors of HRV.\textsuperscript{144} For an in depth phytochemical and pharmacological investigation, it was mandatory to focus on one promising natural material. Thus, we consulted the ethnobotanical source \textit{Materia medica}, which was written by Pedanius Dioscorides.
in the 1st century AD. The treatise consisting of five books comprises some 1,000 or so drugs derived from minerals, animals, and the majority of them from plants (~800). It represents a great repository of botanical, medical, and pharmacological lore.

Scrutinizing the natural materials underlying the obtained virtual hits we came across asafetida, which is a gum resin gained from the roots of a variety of foul-smelling Ferula species from the Apiaceae family. Based on the descriptions given in the Materia medica there is strong evidence that the juice (i.e. resin) of the popular ancient silphion originating from Media and Syria corresponds to asafetida (book III, cap. 80).315 Yielded by incision of the root and stalk and frequently mixed with sagapenon, i.e. the resin of F. persica Willd (book III, cap. 81), it is reported to be effective in the context of upper respiratory diseases, e.g. “for chronic harshness of the throat,” “it clears the voice,” “shrinks the uvulas,” “suitable for a cough,” “for pleurisy,” “for chest pain;” sagapenon is described as follows: “it clears thick matters from the lungs,” “given to those who are chilled.”315 These descriptions finally helped to prioritize those virtual hits, which have been reported to be constituents of asafetida.316 The pharmacological investigation of asafetida and its constituents farnesiferol B and C (Fig. 5; Table III) revealed a distinct anti-HRV-2 effect in the low micromolar range using a CPE inhibitory assay.143 The results of this study provided a rationale for the ancient usage of asafetida for upper respiratory tract infections. On the other hand, the traditionally manifested evidence for asafetida for the treatment of common cold symptoms substantially helped in the selection of this plant material in the search for anti-HRV capsid binders.144

Typically, an ethnopharmacologically based discovery of an active (anti-HRV) extract is followed by a bioassay guided fractionation, and the isolation of those constituents that are responsible for the extracts’ bioactivity. The concept of a bioassay-guided approach is the fractionation accompanied by simultaneous detection of the activity during the separation steps, which results in a continuous enrichment, and finally in the isolation of the active ingredient/s. In this way a large number of anti-HRV agents from natural sources have already been discovered.

Semple et al. investigated the active principle of the Asteraceae plant Pterocaulon sphacelatum, which has been used in traditional medicine of Aboriginal people of Australia as a favored treatment for respiratory infections, especially colds. By means of an antiviral activity-guided fractionation measuring the poliovirus-induced CPE assay, the authors identified the flavonoid 3,7,3'-trimethoxy-5,6,4'-trihydroxyflavone, i.e. chrysosplenol C (Fig. 5; Table III) as potent and specific inhibitor of the picornaviral replication. 6,7,8-Trimethoxycoumarin (Fig. 5; Table III) was also isolated as a major constituent from the ethanolic extract of P. sphacelatum, but showed no activity against poliovirus.317 Interestingly, this coumarin exhibited a significant effect in the HRV-2-induced CPE assay in a recently performed virtual parallel screening study performed in our laboratory.318 In contrast to the isolated coumarin, chrysosplenol C was already known as a member of the 4'-hydroxy-3-methoxyflavones, which represent potent and specific inhibitors of picornaviral, especially rhinoviral replication.319–321 In 1993, Vanden Berghe, Haemers, and Vlietinck provided a profound survey of antiviral agents from higher plants, and demonstrated the impact of ethnobotanical knowledge in their search for antiviral compounds from African medicinal plants. The selection of investigated plant species was mainly based upon their use in the treatment of viral diseases by African traditional healers.320 The antiviral activity of 100 different plant species belonging to 33 families was investigated; thereof 21 species exhibited prominent antiviral properties against one or more of the tested viruses. The most pronounced activity against picornaviruses was recorded within the genus Euphorbia. All compounds detected as antiviral constituents from the respective extracts were identified as 3-methoxyflavone derivatives, especially 3-methylethers of quercetin and kaempferol (Fig. 5; Table III). They showed no significant cytotoxicity and were highly active in tissue culture...
against all human picornaviruses. In tissue culture, cells infected with different picornaviruses (among them also HRV) no CPE was observed, when cells had been treated with 2 μg/mL of these 3-methoxyflavones.

**B. Activity Screening**

The selection of natural materials based on ethnopharmacology is a profound rationale for lead structure discovery and highly superior to random selection. This however rarely applies to marine organisms, fungi, and microbes. Although these organisms are esteemed as highly valuable source for bioactive metabolites, hardly any records from folk medicine are given for them.

A medium-sized activity screening using a robust cell-based or in vitro-assay with reasonable effort as to time and costs is a strategy to get a first insight into the antiviral activities of extracts, fractions, and compounds from synthesis or nature.

For the discovery of novel naturally based HRV 3C-protease inhibitors, Singh et al. used a small peptide containing Q-G scissile bond as substrate for the in vitro screening of extracts. The authors isolated the novel benzoisochromanquinone (+)-thysanone (Fig. 5; Table III) from an extract of the fungus *Thysanophora penicilloides* with potent HRV 3C-protease inhibitory activity. A continued screening revealed a pronounced HRV 3C-protease inhibitory activity for the extract of the Chinese herb *Polygonum cuspidatum*. By bioassay-guided fractionation 2-methoxystypandrone (Fig. 5; Table III), a naphthoquinone, with an IC$_{50}$ value of 4.6 μM, was isolated from the plant material. The total syntheses of this natural compound and further analogues allowed for a structure—activity relationship, and particularly the comparison between activities of ortho- vs. para-quinones. To measure the selectivity of the compound series against cystein proteases other than HRV 3C-protease, the compounds were evaluated against papain. The simple 9,10-phenanthraquinone (Fig. 5; Table III) was the most active compound of the series and showed an IC$_{50}$ value of 1.4 μM with a distinctly higher degree of selectivity than 2-methoxystypandrone.

A CPE reduction assay was applied for the identification of raoulic acid, a bicyclic C25 terpene acid (Fig. 5; Table III) isolated from *Raoulia australis* (Asteraceae). Raoulic acid exerted an antiviral activity against coxsackie virus B3, B4, enterovirus 71, and HRV-2 and -3 with IC$_{50}$ values in the submicromolar range. No activity was recorded against influenza A and B viruses.

In the course of the systematic screening of microbial and natural products for anti-HRV activity, Ishitsuka et al. identified 4',5-dihydroxy-3,3',7-trimethoxyflavone (Fig. 5; Table III) from the leaves of the Chinese medicinal plant *Agastache rugosa* (Lamiaceae) as natural compound with high activity against all picornaviruses except mengovirus. The authors synthesized the orally active 4',6-dichloroflavan (BW863C, Fig. 2; Table II), which revealed an activity against a number of HRV serotypes in the range between 0.007 and 10 μM.

**C. Structure–Activity Relationship**

As soon as an active lead compound is identified it is of utmost importance to scrutinize the derivatives’ activity to (i) obtain insight into the chemical requirements mandatory for the
focused biological activity, (ii) improve the compound’s pharmacological profile in terms of potency, selectivity, cytotoxicity, etc., and to (iii) improve its bioavailability.

The nonphenolic aporphine alkaloid glaucine is a prominent constituent of the aerial parts of *Gaucium flavum* (Papaveraveae). In a recently published study, Spasova et al. investigated the antiviral potential of glaucine (Fig. 5; Table III), glaucine derivatives, and its semi-synthesized 3-aminoethylglaucine cinnamoyl- and hydroxycinnamoyl amides. Beside the anti-oxidative potential of the newly synthesized compounds, they all exerted an antiviral activity against the replication of HRV-14. The best anti-HRV activity was observed for oxoglaucine (Fig. 5; Table III; IC50 $1.5 \mu M$, SI 170), and the $o$-coumaroylamide and $p$-coumaroylamide of 3 aminomethylglaucine (Fig. 5; Table III; IC50 $15 \mu M$, SI 10.3 and IC50 $13 \mu M$, SI 11, respectively).327

The early findings of the anti-HRV active naturally derived 3-methoxyflavone inspired chemists and pharmacologists in the synthesis and the pharmacological evaluation of derivatives thereof decorated with various substitution patterns. Several reports published during the last two decades reviewed the findings of antiviral flavonoid research.320,328,329

By investigating the antiviral activity of a wide variety of naturally occurring flavonoids, Tsuchiya et al. found chrysosplenol B and C (Fig. 5; Table III) contained in *Chrysosplenium* plants, and axillarin (Fig. 5; Table III) as potent anti-HRV agents. Based on their findings, the flavone skeleton decorated with a methoxy group in position 3 and a 5-hydroxyl group revealed as mandatory for an anti-HRV activity.319

A series of antipicornaviral 4′-hydroxy-3-methoxyflavone derivatives was synthesized by De Meyer et al. in order to establish a structure–activity relationship. Thereby, different substitution patterns of the A-ring system with methyl, hydroxy, methoxy, halo, nitro, and amino was performed. Their activity against polio and HRV was compared with those of naturally occurring flavonoids. Further, the importance of the 4′ hydroxyl-group and of the 3-methoxyl-group was confirmed by investigation of different derivatives lacking these features.321 The results showed that 4′-hydroxy-3-methoxyflavones with a monosubstituted A-ring are less active than the corresponding compounds having a polysubstituted A-ring. Within the tested series of compounds, 4′,7-dihydroxy-3-methoxy-5,6-dimethylflavone emerged not only as noncytotoxic but also as most potent substance in both antiviral test systems. The lowest concentrations for this compound that protect 50% of the cells from CPE of 12 HRV serotypes were in the range from 0.016 to 0.5 $\mu g/mL$. In contrast to quercetin, this flavone was also reported to have no mutagenic properties (measured up to 2.5 $\mu g/plate$) in a short-term microbial assay.321

The mechanism studies performed with 3-methoxyflavones have shown an interference with an early stage in the viral RNA synthesis; no induction of resistance was observed.320 In contrast to this mode of action, the anti-HRV chalcones and flavans are reported to interact directly with specific sites on the viral capsid proteins, thereby preventing uncoating and the consequent liberation of viral RNA.193,330,331

Due to its anti-HRV potency, low toxicity, and promising bioavailability, dichloroflavan was evaluated for its protective efficacy against experimental HRV-infection in two clinical, double-blind, placebo-controlled trials. However, the drug candidate failed either when administered orally or intranasally.332,333 Unfortunately, till now no clinical trials evaluating the efficacy of 3-methoxyflavones in common cold have been performed.

### D. Computational Approaches

The common idea of all computational approaches is to extract knowledge from a more or less large set of data in order to make predictions of new events.334 Within the lead discovery process, computational approaches, such as virtual screening, docking, quantitative
structure–activity relationship, have largely enhanced the impact of computational chemistry and nowadays chemoinformatics plays a predominant role in drug research.\textsuperscript{335} The key goal of the use of such methods is to reduce the overall cost associated to the discovery and development of a new drug by identifying the most promising candidates to focus the experimental efforts on. A number of books and reviews on the impact of computational chemistry for lead structure determination highlight these efforts.\textsuperscript{336–339}

In general, in silico methods can be divided into (i) ligand-based approaches, which rely on known active compounds. Based on their physicochemical properties crucial for biological affinity, activities are predicted by extrapolation on not-yet tested substances, e.g. machine learning techniques and classical quantitative structure–activity relationship (QSAR). Ligand-based approaches are invaluable tools in cases where no structural information about the pharmacological targets is available. (ii) On the other hand, structure-based approaches use experimentally determined 3D structures of the targets, such as molecular docking or structure-based pharmacophore modeling for virtual screening. These methods allow for gaining insight into protein–ligand interactions at an experimentally determined (static) level (however not considering flexibility). A unique platform containing 3D coordinates of experimentally solved protein structures (by X-ray crystallography or NMR) is the Protein Data Bank (PDB) currently comprising more than 50,000 structures of biomolecules and protein–ligand complexes.\textsuperscript{340} Additionally, there are several PDB-related web services and tools, which enable to use the PDB-portal in a rich diversity of information services for students and scientists.\textsuperscript{341}

Particularly in the early stage of drug development, such as lead discovery and lead optimization, computational approaches allow for a target-oriented and rationalized proceeding, and thus may substantially help to maximize the success rate.\textsuperscript{338} A recently published review on the impact of computer-assisted approaches in antiviral research thoroughly describes underlying in silico techniques, and highlights the benefits of computational approaches for the discovery of antiviral lead structures.\textsuperscript{342}

In anti-HRV research the capsid protein and the protease 3C revealed to be promising targets (as described before). Inhibitors are assumed to have a major impact for the treatment of HRV-infections. Additionally, these targets are structurally elucidated, and some potent ligands are known as well. These facts enable the performance of sensible computer-assisted approaches, both ligand- and structure-based. Some studies using an in silico approach for the discovery of potential anti-HRV agents focusing on the mentioned targets will be reported in the following paragraphs.

For compounds acting as potential HRV-capsid binders, some classical QSAR and 3D QSAR studies have been performed. While in classical QSAR, the relationship between 2D calculated properties derived from chemical structures and measured biological activities are explored statistically, 3D QSAR techniques are aimed at deriving a correlation and in turn activity prediction based on spatial arrangements of chemical properties and atoms. Applying the 3D QSAR technique CoMFA (Comparative Molecular Field Analysis) statistical models are derived, which are visualized in color-coded contours around the molecule. Therein spots indicate where electrostatic properties and spatial arrangements are favorable for biological activity.\textsuperscript{343}

In the studies of Diana et al., Artico et al., and Verma et al., QSAR techniques helped on one hand to analyze and rationalize the structural features of active compounds essential for the interaction to the HRV canyon’s binding pocket, and on the other hand to search for new classes of capsid binders, thus to narrow the synthetic challenges for specific anti-HRV agents, respectively.\textsuperscript{344–348} In all these investigations hydrophobicity was found to be one of the most important determinants of substance activity. QSAR combined with simplex representation of molecular structure was applied by Kuz’min et al. based on the selectivity
index (CC\textsubscript{50}/IC\textsubscript{50}) and the HRV-2 inhibitory concentration of a set of [(biphenyloxy)-propyl]isoxazole derivatives.\textsuperscript{188} On the basis of QSAR analysis and computational design, three new isoxazoles with high activity prediction were selected and synthesized. They all revealed a strong coincidence between experimental and predicted anti-HRV activity and selectivity index. Terminal benzene substituents with negative electrostatic potential and a molecule length of approximately 5.5–5.6 Å have been suggested as mandatory features within this chemical class for a HRV-2 inhibitory activity.

HRV-serotypes show a high level of conservation at the protease 3C binding site; Sequence alignment and secondary structure predictions suggested an overall architecture and mechanism of HRV-3C proteases that correlates with cellular cystein- and serine proteases, such as chymotrypsin and trypsin.\textsuperscript{349,350} The identity among 3C proteases from different families is however modest and provides space for the development of specific inhibitors for HRV-3C protease.\textsuperscript{350} In a recently published review on selective inhibitors of picornavirus replication, De Palma et al. summarized all currently known chemical structures acting as peptidic or nonpeptidic inhibitors of this viral target.\textsuperscript{156} In 2000, Reich et al., used the HRV 3C protease co-crystal structure information to rationalize the target-oriented synthesis of HRV 3C protease inhibitors from the class of substituted benzamides. Activity data and subsequent crystallographic studies pointed out important requirements for the inhibition of the 3C protease.\textsuperscript{351} Similarly, Maugeri et al. used a structure-based approach, and performed docking studies based on the 3C protease crystal structure with a virtual library consisting of benzamide derivatives. Quantum-mechanic calculation proposed substituents with most promising biological activities. This workflow guided the design and synthesis of substances virtually assumed to act as substrate analogues. Synthesis of some of these compounds and biological testing confirmed the underlying hypothesis.\textsuperscript{219} Quantitative molecular modeling studies were performed to better define and predict interactions between bicyclic 2-pyridone derivatives that showed to be irreversible inhibitors of the 3C protease. In these studies molecular mechanics simulations to evaluate chemical rate of covalent bond formation and free energy calculation combined with crystallographic studies were applied to explain the differences in activity of some irreversible peptidomimetic inhibitors. These data were used as a basis for further optimization of these compounds.\textsuperscript{224,352}

In a recent study performed by Kuo et al. some 6,800 compounds were subjected to a high throughput screening in the search for novel inhibitors for both 3C and 3CL proteases from picornavirus and coronavirus, respectively.\textsuperscript{217} Five nonpeptides were identified with IC\textsubscript{50} values ≤ 10 μM against severe acute respiratory syndrome—coronavirus 3CL-protease; one molecule was found to additionally inhibit the 3C proteases of coxsackievirus, enterovirus, and rhinovirus. This compound (ID 43146) contains a dihydropyrazole ring decorated with two phenyl groups and a lengthy \(N\)-butyl-benzimidazolylamino-toluene. It was used as starting point for the selection of further four analogs showing IC\textsubscript{50} values in the range of 0.5–10 μM against the tested viral proteases. By means of docking-based computer modeling, the authors tried to rationalize the binding discrepancies responsible for individual and common protease inhibitors, thus to provide a rational base for the development of nonpeptide multiple-function inhibitors against coronaviruses and picornaviruses.

\textbf{E. Pharmacophore Models in the Search for Anti-HRV Agents}

In anti-HRV research, first application scenarios have been conducted using pharmacophore models. According to the official IUPAC definition by Wermuth et al.,\textsuperscript{353} a pharmacophore describes the 3D arrangement of steric and electronic features necessary to trigger or block a biological response. Pharmacophores can be represented by three-dimensional chemical
features, which include hydrogen bond donors and acceptors, aromatic rings, hydrophobic groups, as well as positive and negative ionisable moieties. Additionally, the shape of ligands can be represented by shape features, which essentially describe the van der Waals radii of the ligand atoms. The pharmacophore concept has proven to be successful, not only in rationalizing structure–activity relationships but also by its large impact in developing appropriate 3D-tools for efficient virtual screening.\textsuperscript{336,354} Steindl et al. elaborated ligand- and structure-based pharmacophore models implementing the essential feature of the covalent binding to the cysteine 147 in the active site of the HRV-2 3C protease. Thus, the in silico approach focused on defining a new pharmacophore feature representing a target structure for nucleophilic addition in the ligands, which is a crucial step for protease inactivation. The generated hypotheses retrieved known 3C protease inhibitors in the virtual screening cycle, and proposed potential (unconfirmed) ligands of the 3D protease binding site from available databases.\textsuperscript{355}

The viral capsid of several HRVs has been elucidated by crystallization and resolution of the 3D-structure. The HRV coat protein complexed with its highly active inhibitor WIN 61209 was used as starting point for the generation of structure-based pharmacophore models by Steindl et al. in 2005. The models were used for virtual screening of a large commercially available 3D database. For final selection of virtual hits worth to be subjected to biological testing, docking studies and principal component analysis were performed. Six candidates were tested for their ability to inhibit HRV serotype 39 by multiple-cycle CPE inhibition assay. Although all of them showed a certain antiviral potential, one longitudinal piperazine derivative inhibited the virus at a concentration below 10\textsuperscript{–6} M. Some of the test candidates showed difficulties in the interpretation of experimental results due to their relatively high cytotoxicity and bad solubility. This circumstance asks for more cautious estimation of molecular properties for compound selection as stated by the authors.\textsuperscript{356} In a subsequently performed study, the best validated pharmacophore model was used for the identification of naturally derived HRV coat protein inhibitors.\textsuperscript{144} For virtual screening experiments the in-house generated 3D-database DIOS was used (as described before). Based on the virtually predicted ligands and considering knowledge from traditional use, sesquiterpene umbelliferons from the gum resin of Ferula sp., i.e. asafetida, were finally selected as most promising candidates. For biological evaluation, the antiviral activities of asafetida and its isolated constituents were assessed by an exploratory determination of the inhibition of the CPE induced by HRV serotypes 1A, 2, 14, and 16. The results revealed a dose-dependent and selective anti-HRV activity against serotype 2 for asafetida and its virtually predicted constituents, farnesiferols B and C (Fig. 5; Table III; IC\textsubscript{50}: 2.6 and 2.5 \textmu M, respectively). To scrutinize the selectivity of these two compounds against HRV-2 in comparison to the other tested serotypes, the amino acid sequences of HRV-2 and HRV-16 VP1 were aligned. Since all amino acid residues involved in ligand binding showed 100\% match in both serotypes, the experimentally determined selectivity profile could not be explained by different binding pockets. The serotype alignment does however not reflect potential protein flexibility during binding, which might differ between the HRV serotypes. Additionally, off-target effects could be a reason for the observed selectivity.

In a recently performed virtual parallel screening approach, we tried to identify potential targets of human pathological relevance for 16 constituents isolated and identified from the medicinal plant \textit{Ruta graveolens}.\textsuperscript{318} Using the screening platform Pipline Pilot\textsuperscript{357} included in Discovery Studio,\textsuperscript{358} low-energy conformers of the 16 identified molecules from \textit{R. graveolens} were subjected to parallel screening. For this purpose, the Inte:Ligand pharmacophore model collection was used.\textsuperscript{359} It currently comprises 2,208 models covering 280 unique pharmacological targets. Based on the predicted ligand-target interactions, the authors focused on three biological targets, namely acetylcholinesterase, the HRV coat protein, and
the cannabinoid receptor type 2. Virtual hits and nonhits were assayed on their respective targets for a critical evaluation of the performed target-fishing approach. Beside other predicted bioactivities, determination of their CPEs on HRV-2 revealed the virtual hit arborinine (Fig. 5; Table III; IC50: 3.2 μM) and the nonpredicted hit 6,7,8-trimethoxycoumarin (Fig. 5; Table III; IC50: 12.9 μM) as the most active anti-HRV constituents. It could be shown that the applied in silico strategy has the capacity of catalyzing drug discovery profoundly for all those diseases where molecular targets or molecular ligands are well defined to create reliable pharmacophore models.

7. CONCLUSION

HRV, a prominent member of the Picornaviridae family, infects humans more frequently than any other virus. Infections with HRV mainly lead to upper respiratory diseases, such as the common cold, but may also cause more severe lower respiratory tract disorders. Although the symptomology and severity of the common cold is relatively mild and the course of disease self-limiting, the socio-economic impact is tremendous in terms of recouping lost productivity due to sick leave.

In the last decades, a number of anti-HRV agents from different origin—synthetics, natural compounds, biologicals, botanicals, and nutritionals—have been discovered. Different concepts have been used as strategy for their discovery either starting from a phenomenological effect, e.g. empirical knowledge from folk medicine, or from a target-based molecular level, e.g. ICAMs, capsid binders, and HRV protease inhibitors. Some of the outcomes revealed potent and promising activities that partly have been evaluated for the management of HRV-induced common colds in clinical trials mainly with sobering benefit. Since the HRV infection is not life-threatening in most cases, a potential therapy has to be safe and effective with an almost unrecognizable level of side effects. These preconditions render the search for anti-HRV-agents into a high challenging endeavor and explains why no antiviral agent is approved for the prevention or treatment of HRV-infection until today, despite the significant efforts. Moreover, the high variability of rhinoviruses suggests the need of more than one active principle covering different modes of action for an effective treatment. Therefore, there is a high need for an ongoing search for new synthetic as well as natural compounds on a molecular level.

The increasing knowledge about the HRV life cycle, gene, and protein sequence combined with the improved technologies in the field of experimental and computational methods have continuously enabled further insights into the spectacular world of HRV. Only with this fundamental research, virtual screening approaches, such as QSAR, pharmacophore- and docking-based screening cycles, or computational compound design, are applicable on a rational base. With the gained expertise from different disciplines and an adequate infrastructure for further research, it is encouraging to hope that discovery and clinical development efforts will continue in the search for agents that may treat or prevent the annoying common cold.

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