

# *Selective Human Enterovirus and Rhinovirus Inhibitors: An Overview of Capsid-Binding and Protease-Inhibiting Molecules*

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**Abstract:** The absence of effective vaccines for most viral infections highlights an urgent necessity for the design and development of effective antiviral drugs. Due to the advancement in virology since the late 1980s, several key events in the viral life cycle have been well delineated and a number of molecular targets have been validated, culminating in the emergence of many new antiviral drugs in recent years. Inhibitors against enteroviruses and rhinoviruses, responsible for about half of the human common colds, are currently under active investigation. Agents targeted at either viral protein 1 (VP1), a relatively conserved capsid structure mediating viral adsorption/uncoating process, or 3C protease, which is highly conserved among different serotypes and essential for viral replication, are of great potential to become antipicornavirus drugs. © 2004 Wiley Periodicals, Inc. *Med Res Rev*, 24, No. 4, 449–474, 2004

**Key words:** picornavirus; enterovirus; rhinovirus; capsid protein VP1; 3C protease

## **1. INTRODUCTION**

Enteroviruses and rhinoviruses are the common cause of infections in human. The human enteroviruses caused various illnesses, some easily recognized clinically while others diagnosed as non-specific viral syndromes. The severity of enterovirus infection depends mainly on the somatopic localization after primary replication in gastrointestinal tract and subsequent bloodborne dissemination. Clinical manifestations of enterovirus infections include fever alone, or specific

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syndromes such as hand, foot, and mouth disease, and herpangina. However, the same viruses also cause potentially severe and life-threatening infections such as meningitis, encephalitis, myocarditis, polio-like syndrome, and neonatal sepsis. Human rhinoviruses infections cause nasopharyngeal syndrome (the “common cold”) in general population of all ages. Although rhinovirus infection is self-limited, complications could still occur in patients with asthma, congestive heart failure, bronchiectasis, and cystic fibrosis.

To date, no antiviral agent has been approved by FDA for the treatment of either enterovirus or rhinovirus infection. Clinical treatments are directed toward symptomatic relief of the most prominent symptoms of each clinical syndrome. Steps such as viral attachment, uncoating, viral RNA replication, and protein synthesis in the replication cycle of enteroviruses/rhinoviruses can serve as potential targets for antiviral agents. The following sections briefly review the virology and clinical diseases of enteroviruses and rhinoviruses, and the capsid-binding/protease-inhibiting molecules that are potential agents for drug development.

## 2. VIROLOGY

Enterovirus and rhinovirus are two important pathogens within the family of picornaviridae. The human enteroviruses, so-called because most inhabit the enteric tract, include the polioviruses (types 1, 2, and 3), coxsackieviruses A (23 serotypes), coxsackieviruses B (6 serotypes), the echoviruses (32 serotypes), and the numbered enteroviruses 68–73 (Table I).<sup>1–4</sup> Enterovirus 72 has been reclassified as the hepatitis A virus.<sup>5,6</sup> The rhinoviruses, so-called because of their special adaptation to the nasopharyngeal region, are the most important etiologic agents of the common cold in adults and children. There are more than 100 serotypes of rhinoviruses in existence.

### A. Viral Genome and Replication

The picornaviral genome consists of a single-stranded, positive sense (messenger-active) RNA. This viral RNA has a small protein called VPg covalently attached to its 5'-end and is polyadenylated at its 3'-terminus.<sup>7,8</sup> The genomic RNAs vary in length from 7,200 to 7,500 bases.<sup>7,9–13</sup> The 5'-non-coding region (5'-NCR) is long and highly structured, containing a cloverleaf-like structure that is important for negative strand viral RNA synthesis<sup>14,15</sup> and an internal ribosome entry site (IRES) that is essential for directing translation of mRNA.<sup>16–18</sup> The 3'-NCR is short, ranging in length from 47 to 125 bases. The 3'-NCR also contains a secondary structure, notably a pseudoknot that plays a role in controlling viral synthesis.<sup>19</sup> Replication of picornaviruses takes place entirely in cytoplasm. After attachment to the host cell, the viral genomic RNA is uncoated from the viral capsid. The positive stranded viral RNA is translated to viral proteins that are essential for viral gene replication and production of new viral particles. Genome replication and mRNA synthesis occur in small membranous vesicles that are induced by several viral proteins. A single replication cycle ranges from 5 to 10 hr. The speed of viral

**Table I.** Members of Enteroviruses

<i>Subgroup</i>	<i>Serotypes</i>
Poliovirus	1–3
Coxsackievirus A	1–22, 24
Coxsackievirus B	1–6
Echovirus	1–9, 11–27, 29–31
Numbered enteroviruses	68–71, 73

Coxsackievirus A 23 has been reclassified as echovirus 9. Echovirus 10 and 28 have been reclassified as reovirus 1 and rhinovirus 1A. Enterovirus 72 has been reclassified as hepatitis A virus.

replication depends on many factors, such as virus strain, environmental temperature, pH, host cell type, and multiplicity of infection.

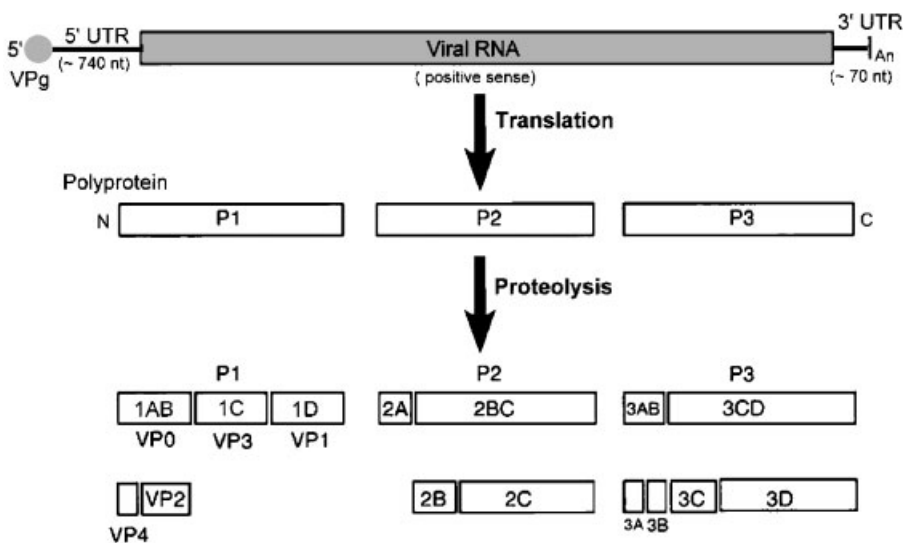
### B. Viral Capsid Proteins

Picornavirus virions are spherical in shape with a diameter of about 40 nm. The viral particle has no lipid envelope. Enteroviruses are acid stable and retain infectivity at pH lower than 3.0. Rhinoviruses, in contrast, are labile at pH less than 6.0. The capsids of picornaviruses are composed of four structural viral proteins, namely, VP1, VP2, VP3, and VP4. The capsid contains 60 structural proteins arranged into an icosahedral lattice.<sup>20,21</sup> The basic building block of the picornaviral capsid is the protomer, which contains one copy of each VP1, VP2, VP3, and VP4. The shell is formed by VP1, VP2, and VP3, VP4 lies on its inner surface. VP1, VP2, and VP3, though with no homology in sequence, form a common structure: the  $\beta$ -barrel jelly roll. The main structural difference between VP1, VP2, and VP3 is the loop that connects the  $\beta$ -strands and the N- and C-terminal sequences that extend from the  $\beta$ -barrel domain.<sup>22</sup> The amino acid sequences give each picornavirus its distinct antigenicity. The surface of the virion has a prominent star-shaped plateau at the fivefold axis of symmetry, surrounded by a deep depression (“canyon”). It has been proven, in poliovirus and rhinovirus, that the canyon serves as a receptor-binding site.<sup>23–26</sup>

### C. Viral Proteases

The viral RNA is translated into a long polyprotein. This single polyprotein then undergoes proteolysis by virus-encoded protease 2A and 3C (Fig. 1). Cleavage of the Tyr–Gly pairs which connect coat precursors P1 to P2–P3 and 3C’–3D’ in enterovirus is accomplished by viral proteinase 2A,<sup>27</sup> but the cleavage of 3C’–3D’ by protease 2A is not essential for viability of the virus.<sup>28</sup> The remaining cleavage in P2–P3 at Gln–Gly pair is executed by viral protease 3C, which is essential for enterovirus replication.<sup>29,30</sup> Sequence alignment for enterovirus 3C protease reveals no homology with mammalian protease. Therefore, 3C protease is a potential target for drug discovery.

In addition to the cleavage of viral polyprotein, it has been shown that 2A<sup>pro</sup> cleaves the host cell protein eIF4G.<sup>31–35</sup> Cleavage of eIF4G prevents eIF4F from recruiting 40S ribosomal subunits to capped mRNAs, because the cleavage releases the N-terminal domain of eIF4G which binds to eIF4F



**Figure 1.** Proteolytic processing of enterovirus polyprotein.

which in turn binds to the 5' cap of cellular mRNA. This event shuts off the translation of host cellular mRNA. The viral proteases 2A and 3C also contribute to poliovirus-induced apoptosis.<sup>36–38</sup> Poliovirus 2A induces apoptosis through the cleavage of translation initiation factor eIF4G,<sup>36</sup> whereas poliovirus 3C kills cells by apoptosis through the activation of caspase.<sup>38,39</sup> Similar apoptotic pathways have also been demonstrated in enterovirus 71 (EV71). Transient expression of EV71 2A protease results in triggering apoptosis.<sup>40</sup> EV71 3C protease induces apoptosis in the human neural cells via the activation of caspase.<sup>41</sup>

### 3. CLINICAL DISEASES

Rhinoviruses usually cause “common cold,” while enteroviruses may cause very different clinical manifestations as listed in Table II.

#### A. Respiratory Illness

The common cold (summer cold), pharyngitis, tonsillitis, and croup have been frequently reported.<sup>42</sup> Most of the respiratory illnesses caused by human rhinovirus and enterovirus are benign, but symptoms may persist for several days, and the resultant interruption in school and work days may be substantial. In an etiology study of viral respiratory illness, rhinoviruses infection has been found to be the top cause (35.8%).<sup>43</sup> The enteroviruses are responsible for approximately 15% of upper respiratory infections for which etiology is identified.<sup>44,45</sup> Group A coxsackieviruses are the most common cause of herpangina. However, coxsackie B viruses and echoviruses have also been reported to have the same clinical manifestations.<sup>46</sup> Children 1- to 7-year-old are the group with the highest incidence. There is usually an abrupt onset of fever associated with sore throat, dysphagia, and malaise. Grayish white vesicles can be seen in the posterior portion of the palate, uvula, and the tonsillar pillars. The fever lasts for 1–4 days and the symptoms begin to improve in 4–5 days, and recovery is usually within 7 days of onset.

#### B. Hand-Foot-and-Mouth Disease (HFMD)

HFMD is one of the common diseases in children, especially the children under 4 years of age. The disease is usually mild, and the onset is associated with a sore throat with or without fever. Scattered vesicular lesions can be observed in the mouth, hands, feet, and hip. EV71 and coxsackievirus A 16 are closely related in genetics and both are causative agents of HFMD. However, EV71 is associated with

**Table II.** Diseases Caused by Enteroviruses

Syndrome	Viruses
Respiratory illness	Coxsackieviruses A21, A24; Echovirus 11,20; coxsackievirus B; others
Hand-foot-and-mouth diseases	Coxsackieviruses A9, A16
Meningitis/encephalitis	Many enteroviruses; enterovirus 71; echoviruses
Poliomyelitis	Polioviruses 1,2 and 3
Cardiovascular diseases	Coxsackievirus B; some coxsackieviruses A and echoviruses
Hemorrhagic conjunctivitis	Enterovirus 70; coxsackievirus A24

severe neurological disease, such as encephalitis, meningitis, poliomyelitis syndrome, and even fatal pulmonary edema.<sup>48–52</sup> The neurovirulence of EV71 first came to attention in 1975 in Bulgaria when 44 people died of a polio-like disease.<sup>53</sup> Epidemics of EV71 causing CNS disease subsequently occurred in New York, Australia, Europe, and Asia.<sup>47,54–58</sup> An unusual epidemic of HFMD complicated with fatal myocarditis and pulmonary edema occurred in Malaysia in 1997, and EV71 was implicated as the etiology of the outbreak.<sup>50</sup> In 1998, there was a large scale of HFMD outbreak in Taiwan. Cox A 16 was presumed to be the cause of HFMD at the beginning of this enterovirus epidemic. However, numerous severe complications were later found to follow cases of HFMD and 78 patients died rapidly during this outbreak. EV71 proved to be the major cause of this HFMD outbreak.<sup>51,52,59</sup>

### **C. Enteroviral Meningitis and Encephalitis**

Enteroviral meningitis is the most common cause of aseptic meningitis, and occurs in 4.5–30 per 100,000 population annually with a duration of illness lasting between 7 and 14 days.<sup>60–62</sup> Coxsackievirus B was associated with aseptic meningitis in 62% of infants.<sup>63</sup> Echovirus is a very important pathogen for meningitis.<sup>64</sup> The onset of enteroviral meningitis is usually sudden, with high fever of 38–40°C. The fever pattern may be biphasic. Symptoms and signs may include headache, nausea, vomiting, stiff neck, myalgia, rash, and muscle weakness. Aseptic meningitis caused by certain enterovirus serotypes is associated with particular clinical stigmata. Encephalitis due to enterovirus infection is also well documented. Unlike aseptic meningitis, enteroviral encephalitis may have more profound acute disease and long-term sequelae.<sup>65</sup> The illness usually begins like aseptic meningitis, with fever and other symptoms. Central nervous system (CNS) signs include confusion, weakness, lethargy, drowsiness, and irritability. Coma or seizures may also occur. Enteroviral meningitis/encephalitis usually has a good prognosis. However, as mentioned previously, EV71 meningitis/encephalitis may accompany pulmonary edema and leads to fatality.<sup>48–52</sup>

### **D. Poliomyelitis**

With the great success of the poliovirus vaccination program, poliomyelitis has now been eliminated from most of the world. When polio was widespread, most of the wild-type poliovirus infections are asymptomatic and only 0.1% of poliovirus infections result in paralysis. The remaining infections caused mild flu-like illness. The paralytic manifestations of poliovirus infections reflect the regions of CNS severely affected.<sup>66,67</sup> The overall mortality rate of spinal poliomyelitis is about 5%; bulbar and medullary poliomyelitis are of higher mortality rate (near 50%).

### **E. Cardiovascular Diseases**

The enteroviruses are the common pathogens that cause acute myocarditis.<sup>68</sup> Neonates and young infants are particularly susceptible to coxsackievirus B virus-associated myocarditis. RT-PCR-based studies of endomyocardial biopsies and autopsy specimens revealed that enteroviruses were the cause of acute myocarditis. Symptoms include palpitations, chest pain, and fever. Most of the patients recover uneventfully while small percentage of patients develop congestive heart failure, chronic myocarditis, or dilated cardiomyopathy.<sup>69,70</sup>

### **F. Hemorrhagic Conjunctivitis**

Among enteroviruses, enterovirus 70, and coxsackievirus A 24 are the most common pathogens causing hemorrhagic conjunctivitis. Clinical manifestations caused by these two enteroviruses are indistinguishable, including eyelid swelling, lacrimation, and pain in the eyes. Recovery is usually complete within 1–2 weeks after onset while rare cases develop a poliomyelitis-like illness.<sup>71–74</sup>

#### 4. SMALL MOLECULES AGAINST RHINOVIRUSES AND ENTEROVIRUSES

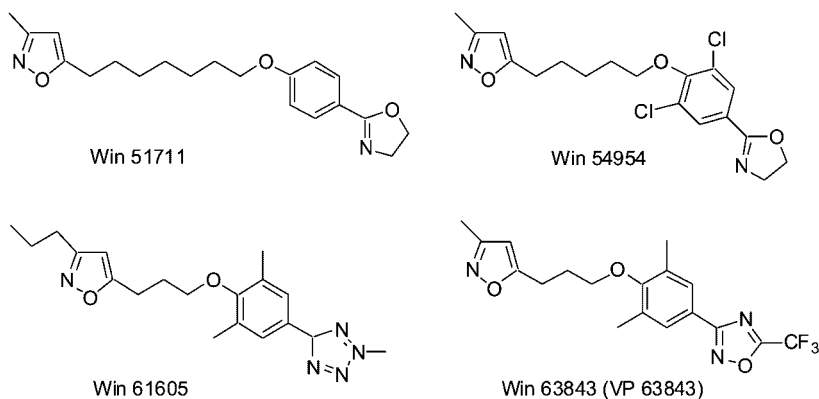
For some viral infectious diseases, such as those infected by poliovirus, hepatitis B virus (HBV) and influenza virus types A and B, vaccination appears to be an efficient and feasible way for disease prevention. For human rhinoviruses and enteroviruses, however, this protocol may be difficult to follow due to a broad spectrum of variants. To date, at least 102 and 65 distinct serotypes, respectively, for human rhinoviruses and enteroviruses have been reported. In addition to numerous variants, the high-mutation rate during viral replication also presents a formidable challenge for the development of effective vaccines. For these reasons, effective antiviral drugs to treat diseases caused by infection of rhinoviruses and enteroviruses should not only possess high potency and low toxicity but have also a broad spectrum of activity.

In common with many other viral pathogens, several steps in the life cycle of picornaviruses, including initial attachment, RNA polymerization, and polyprotein processing, could be targeted for potential antiviral therapy. Over the past two decades, inhibiting viral attachment/uncoating by VP1 blockers and interrupting viral replication via targeting 3A coding region or 3C protease have all been attempted in order to find effective antipicornaviral agents. However, these efforts have met with little success up to this point. Despite the disappointing results, the capsid protein VP1 is considered the most promising therapeutic target due to the fact that pleconaril, a drug candidate of the Win series with potential use in fighting cold, has reached a very advanced stage (Phase III–IV) in its clinical trials. Although pleconaril was finally rejected because of safety concerns, it is believed that the underlying cause of the adverse side effects is structure-based rather than target-based in nature. The remaining sections of this review will focus on the current efforts in developing small-molecule antiviral agents, with a particular emphasis on chemical structures exerting biological activities on either VP1 capsid protein or 3C protease.

##### A. Capsid-Binding Agents

Capsid-binding molecules block viral infection by inhibiting viral uncoating and/or viral attachment to cellular receptors on host cells. The binding site for capsid-binding compounds appears to be a hydrophobic pocket inside VP1 located under the canyon floor. Between the floor and pocket is a section containing the GH loop, a region displaying the greatest changes in viral structure induced by compound binding. Two hypotheses have been proposed to explain how capsid binders mediate antiviral functions mentioned above. In terms of uncoating, insertion of a compound into the VP1 hydrophobic pocket leads to an increase in the stability of the viral particle, rendering the virus more resistant to uncoating, a process necessary for the release of viral RNA. It is believed that uncoating of viral particle requires certain degree of capsid flexibility. Interaction with capsid binders may produce a more compacted capsid structure with limited vacant space for conformational changes essential for uncoating to take place. As for attachment, binding of inhibitors to the VP1 pocket may induce a conformational change in the viral canyon floor, the binding site of cellular receptors such as ICAM-1 molecule identified as the major rhinovirus receptor, and thus prevent adsorption of the viruses to the host cells.

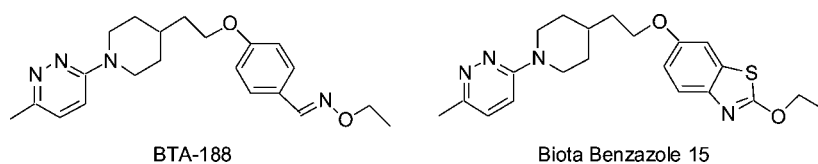
Among capsid-binding compounds, the Win series of compounds in Figure 2 play a remarkable role in the development of antiviral agents against both rhino- and entero-viral infections. Disoxaril, also known as Win 51711, was the first compound of this family with satisfactory biological profiles to enter the clinical trial. This compound was found to be effective *in vitro* against most rhino- and enteroviral serotypes tested.<sup>75–79</sup> It also showed oral efficacy in preventing poliovirus-2 and echovirus-9 induced paralysis in mice. However, its clinical studies were discontinued due to the appearance of crystalluria in healthy volunteers at high dosage. A successor, Win 54954, was subsequently evaluated in Phase II for *in vivo* efficacy against two rhinoviruses (RV 23 and 39) and one enterovirus (coxsackievirus A21).<sup>80–82</sup> The compound significantly attenuated viral titers and the



**Figure 2.** Structures of Win series compounds.

severity of colds induced by coxsackievirus A21 but failed to show any efficacy against either rhinovirus. Win 54954 had a very short half-life, presumably due to the acid lability of the oxazoline ring, and was not further developed for clinical use owing to adverse effects of flushing and rash. Attempts were then made to discover more hydrolytically stable analogues with comparable antiviral potency. These studies led to the discovery of a series of 2-methyltetrazole compounds which are not only resistant to acidic conditions but maintain a broad spectrum of activity.<sup>80,83</sup> Win 61605, regarded as the most promising candidate in the series, was selected for the treatment of rhino- and entero-viral infections. Unfortunately, when administered orally to beagles, this molecule caused hepatotoxic side effects. The hepatotoxicity is presumably due to the multiple nitrogen tetrazole ring or its metabolic product(s). As a result, Win 61605 was dropped for further evaluation. In continuation of the search for structurally related bioisosteric molecules with reduced hepatotoxicity, Win 63843, also referred to as pleconaril, finally emerged as a promising new drug candidate for the treatment of human enteroviral infections.<sup>84–86</sup> In addition to a better metabolic stability in the monkey liver microsomal assay, the newly developed 5-methyl-oxadiazole analogue has also been shown to be more potent than its oxazoline (Win 54954) and tetrazole (Win 61605) predecessors against a variety of rhino- and entero-viruses. Pleconaril can be given by oral administration and is currently being developed by ViroPharma for the treatment of diseases associated with picornavirus infections. This drug candidate is in its late-stage clinical trials for treating viral respiratory infections and viral meningitis. Unfortunately, even though pleconaril was demonstrated to be effective in shortening the number of days patients felt sick and reducing the severity of symptoms, it was not approved by FDA for marketing due to safety concerns, making the drug's fate uncertain. Considering the tremendous synthetic efforts made as well as the two decades consumed on the development of the Win compounds, this unexpected result is extremely discouraging.

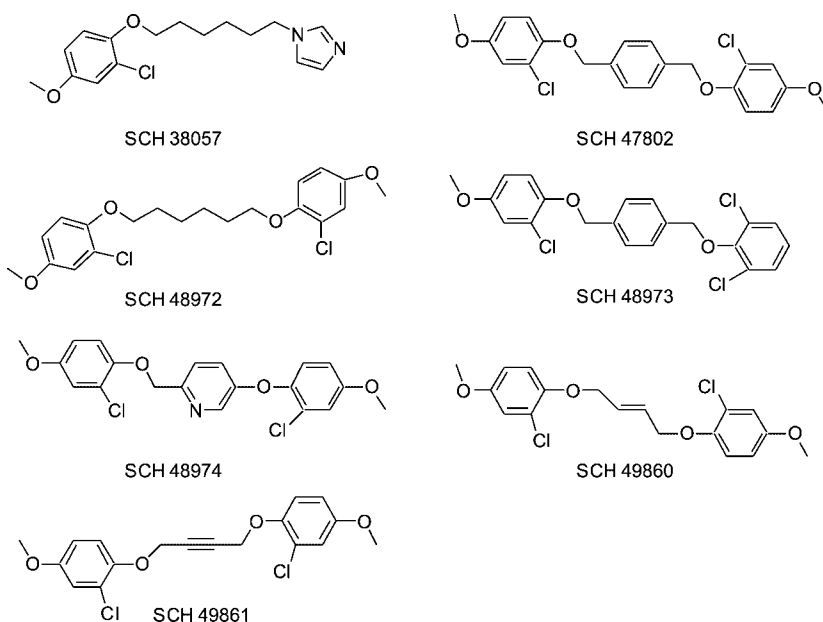
BTA-188 (Biota Scientific Management Pty. Ltd.), a lead of a new class of capsid-binding antiviral agents, has been shown to possess a broad spectrum of activity against rhinoviruses.<sup>87–90</sup> BTA-188 (Fig. 3) inhibits 87 of 100 HRV serotypes. In the cytopathic effect reduction assay for HRV-14, BTA-188 ( $EC_{50} = 1.0$  ng/mL) was found to be superior to both pleconaril ( $EC_{50} = 30$  ng/mL)



**Figure 3.** Structures of Biota.



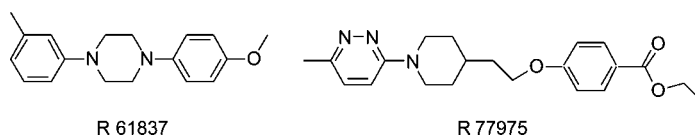




**Figure 5.** Structures of Schering–Plough.

the survival of infected mice when it was administered orally at dosages of 3–20 mg/kg/day. All untreated animals died at day 14. Interestingly, SCH 48974, an analogue with a slight difference in structure from SCH 47802, only showed a marginal effect on survival at a dose of 90 mg/kg/day. The other three derivatives (SCH 48972, 49860, and 49861), although possessing moderate activity against poliovirus 2 in *in vitro* assay, failed to show *in vivo* efficacy. As for mechanisms of action, time-course studies with SCH 47802 and 48973,<sup>96</sup> the two most potent compounds in this series, revealed that they acted on the early adsorption/uncoating step of the poliovirus infection. The results are not unexpected, considering these are linear hydrophobic molecules with considerable structural similarity to the Win compounds. No clinical information is available for this class of compounds up to the present.

Pirodavis (R 77975) and its predecessor (R 61837), as shown in Figure 6, were discovered at Janssen Research Foundation. Both compounds possessed significant activity in inhibiting the replication of many rhinovirus serotypes.<sup>98–100</sup> Compared to R 61837, pirodavis showed an improvement in potency by more than 500-fold *in vitro* and inhibited about 80% of rhinoviral serotypes at concentrations of 0.1 µg/mL or less. When the nasal sprays were given six times a day for 5 days to the patients, significant reductions in virus shedding occurred but no clinical benefits were observed. The lack of clinical efficacy of pirodavis could be due to the low-water solubility of this series, making it difficult to administer in an aqueous formulation compatible with respiratory secretion, and/or the labile ester functional group prone to rapid hydrolysis to form the corresponding inactive acid. Although R 61837 is much less active against most rhinovirus serotypes relative to R 77975, when given prophylactically, it was found to be effective in preventing colds in human



**Figure 6.** Structures of Janssen Research Foundation.

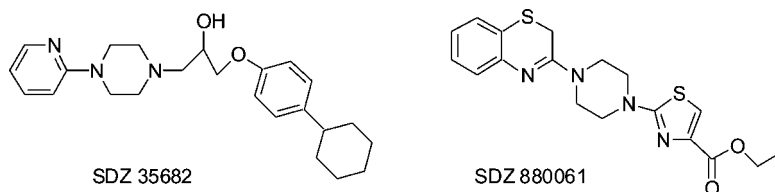


Figure 7. Structures of Sandoz Forschungsinstitut.

volunteers challenged with rhinovirus 9. Given by intranasal spray sixtimes a day for 4 consecutive days (total dose, 25 mg) commencing 28 hr before virus challenge, R 61837 was able to suppress symptoms until 48 hr after medication ceased. In these studies, both compounds were formulated with 10% 2-hydroxypropyl- $\beta$ -cyclodextrin to enhance their water solubility.

Independently, a class of compounds which shared the common piperazine-ring motif with R 61837 was discovered at Sandoz Forschungsinstitut.<sup>101–103</sup> As typified by SDZ 35682 and 880061 in Figure 7, these novel piperazine-containing derivatives are potent and selective inhibitors against a series of human rhinovirus serotypes and some enteroviruses *in vitro*. The former (SDZ 35682) is active against rhinovirus serotypes such as HRV 14, 26, 35, 37, 43, and 48 with  $IC_{50}$  less than 0.1  $\mu\text{g}/\text{mL}$  and echovirus 9 with  $IC_{50}$  value of 0.3  $\mu\text{g}/\text{mL}$ ; the latter (SDZ 880061) is more selective towards human rhinoviruses with a relatively broader antiviral spectrum. Of the 89 HRV serotypes tested, SDZ 880061 inhibited 31 in 89 with  $IC_{50}$  values equal to or lower than 0.0003  $\mu\text{g}/\text{mL}$  and 76 in 89 with  $IC_{50}$  lower than 3  $\mu\text{g}/\text{mL}$ . Similar to the R series, SDZ 35682 and 880061 are also typical capsid-binding molecules, the evidence of which was individually substantiated by their co-crystallization with HRV 14.<sup>102,103</sup> A considerable conformational change at VP1 binding site was observed in the HRV 14/SDZ 35682 complex in which SDZ 35682, a compound of 19 Å in length, fills the entire VP1 hydrophobic pocket including the innermost end and occupies the space more efficiently than other long antiviral agents such as Win 51711. It has been suggested that compounds fitting into the entire pocket might affect the uncoating process of the viral particles. SDZ 880061 was also found to bind at the same pocket in its HRV 14 complex structure. However, the innermost portion of the pocket is vacant, causing less alteration of the VP1 backbone conformation compared to other antiviral agents analyzed structurally. As a result, SDZ880061 only has marginal effects on viral uncoating. This may provide an explanation for the observation that, in the time-course studies, SDZ 880061 was found to primarily interfere with the HRV 14 adsorption to the cell instead of inhibiting viral uncoating. Both of these compounds showed no detectable cytotoxic effect up to 30  $\mu\text{g}/\text{mL}$ . SDZ35682 at a dose of 126 mg/kg reduced echovirus 9-induced paralysis and shortened the mean time of paralysis by 70% in mice. Greater than 85% protection from Echovirus 9-induced death can be achieved by either a low dose (71 mg/kg) given for 6 days or a high dose (126 mg/kg) administered for 2 days. Although SDZ 35682 showed antiviral efficacy in mice, its clinical usefulness may be limited due to a narrow antiviral spectrum. As for SDZ 880061, its *in vivo* studies are not available.

Some capsid function inhibitors are synthetic derivatives or analogues based on the core structures of naturally occurring products. Rhodanine (Fig. 8), 2-thio-4-oxothiazolidine, was synthesized and evaluated in various biological systems in the 1970s.<sup>104,105</sup> The results revealed that

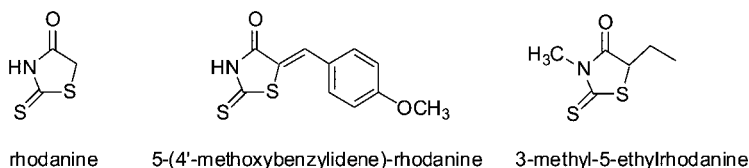


Figure 8. Structures of rhodanine and its analogues.

the spectrum of the virus inhibitory activity of rhodanine was extremely narrow. At a concentration of 12.5  $\mu\text{g/mL}$ , only selective inhibition against echovirus 12 was observed. However, it is non-toxic to the uninfected host cells (monkey kidney cells) at a concentration up to 150  $\mu\text{g/mL}$ . Several derivatives and analogues of rhodanine were prepared and tested (Fig. 8), but they were all considerably less potent than rhodanine itself.<sup>104</sup>

4',6-Dichloroflavan (BW 683C) as illustrated in Figure 9 with a flavanoid-like skeleton is highly effective against some of the most prevalent rhinoviral serotypes (1A, 1B, 2, 15, 29, and 31), with *in vitro*  $\text{IC}_{50}$  values between 0.007 and 0.17  $\mu\text{M/mL}$ .<sup>106</sup> Mechanistic studies indicated that BW683C blocked viral replication by inhibiting a stage immediately after the entrance of the viral RNA into host cells. In order to improve the potency as well as to broaden the antiviral spectrum, synthetic flavanoids substituted with halo, cyano, and amidino groups were prepared and tested for their *in vitro* activity against HRV 1B, polio 2, cox B4, echo 6, and EV71.<sup>107–109</sup> Among the synthetic flavanoids tested (e.g., compounds **1–4** shown in Fig. 9), 4'-chloro-6-cyanoflavan (**3**) was found to be not only more active against HRV 1B infection than parental BW 683C, but also showed good antienteroviral activity within micro and submicromolar range ( $\text{IC}_{50} = 0.32\text{--}1.28 \mu\text{M}$ ). In contrast, BW 863C was inactive for most of the enteroviruses tested with the exception of cox B4 for which moderate activity was observed ( $\sim 2.6 \mu\text{M}$ ). Among the compounds listed in Figure 9, compound **3** exhibited the most potent activity against EV71 ( $\text{IC}_{50} = 0.45 \mu\text{M}$ ). However, relative to those EV71-specific imidazolidinones (Fig. 4) discovered at NHRI (Taiwan), this compound is much shorter in length with a decrease in activity by tenfold. The higher potency observed for the imidazolidinones could be attributed to their more efficient occupation of the VP1 pocket to produce structurally more stable virions.

4'-Ethoxy-2'-hydroxy-4,6'-dimethoxychalcone (Ro 09-0410) in Figure 10 is a chalcone-like synthetic compound which possesses significant activity against rhinoviruses, but shows no activity against other picornaviruses.<sup>110,111</sup> Among 53 rhinovirus serotypes tested, 46 were sensitive to Ro 09-0410 in HeLa cell cultures. The  $\text{IC}_{50}$  value for antiviral activity is around 0.03  $\text{mg/mL}$  while the  $\text{CC}_{50}$  (50% cytotoxic concentration) is more than 30  $\text{mg/mL}$ . Ro 09-0410 was found to be ineffective against rhinovirus infections in human volunteers, probably because of its poor water solubility and oral bioavailability. Studies on various analogues related to this antirhinovirus agent led to the identification of a novel class of chalcone amide analogues<sup>112</sup> (e.g., 09-0696, 09-0881 in Fig. 10) which, compared to Ro 09-0410, were 4.5- to 10-fold more active against 12 selected HRV serotypes at concentrations as low as 2–3  $\text{ng/mL}$  and showed very low cytotoxicity (30–50  $\text{mg/mL}$ ). Labeling studies indicated that these amide compounds competitively inhibited the binding of Ro 09-0410 [ $^3\text{H}$ ] to the viral capsid site in a manner similar to BW 863C, VP 63843, and WIN 51711. No clinical information is available for this series of compounds.

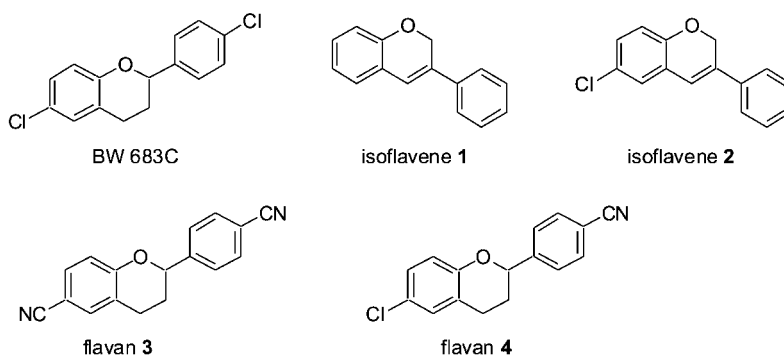
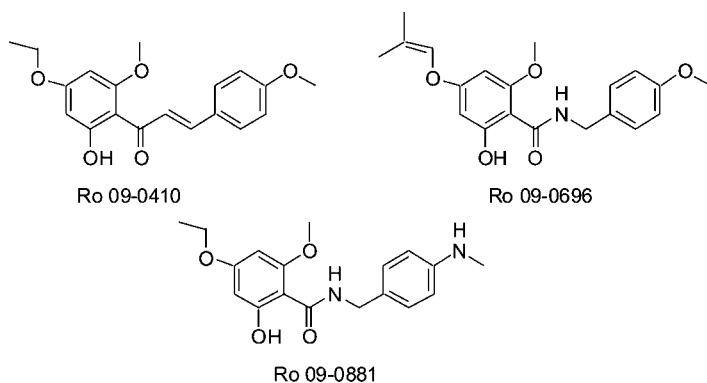


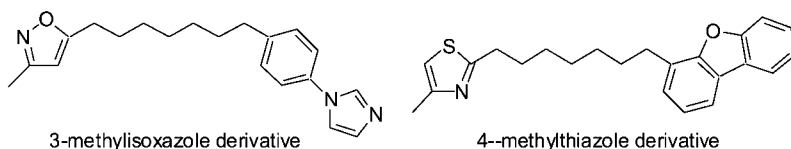
Figure 9. Structures of flavanoid-like analogues.



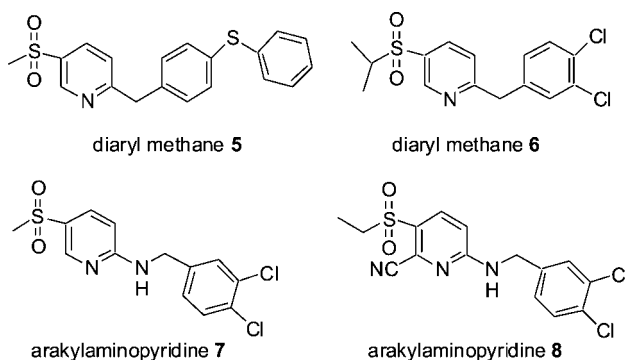
**Figure 10.** Structures of chalcone and chalcone amides.

The mode of action of above-mentioned capsid-binding molecules has been experimentally verified with evidence indicating that the inhibition of viral replication occurred at the early stages during viral attachment and/or uncoating. Some compounds, while their mechanisms have not been studied or reported, are also likely to function as capsid-binding molecules in light of their linear structural profiles as well as lipophilic properties. For example, a novel class of azolyalkyloxy compounds synthesized by Synphar Laboratories, Inc., Edmonton, Alberta, Canada is believed to mimic Win 51711 (disoxaril).<sup>113</sup> The active compounds of this series, as typified with 3-methylisoxazole and 4-methylthiazole derivatives in Figure 11, showed *in vitro* activity against 33–40 of a panel of 52 rhinovirus serotypes with  $IC_{50}$  values ranging from 0.5 to 25 mg/mL. Some of them also showed moderate activity ( $IC_{50} = 1–25 \mu\text{g/mL}$ ) against 5 to 6 serotypes of 7 enteroviruses tested. Moreover, the structure–activity relationship studies revealed that, like most of Win compounds, the optimal length of the alkyl chain between two terminal heterocyclic moieties of this series is either 6 or 7 methylene units.

In sharp contrast, some antipicornaviral agents, such as the diaryl methanes and arakylamino-pyridines shown in Figure 12 and the 2-(4-pyridylaminomethyl)benzimidazole derivatives shown in Figure 13 possess a much shorter chain length in which a linker containing only one or two atoms is observed. The first two classes, diaryl methanes and arakylaminopyridines, were developed by Kenny et al.<sup>114</sup> Among these 26 compounds evaluated against rhinoviruses 1A, 2, and 64 as well as coxsackieviruses 21, several were found to exhibit moderate activity with  $IC_{50}$  ranging from 0.3 to 5  $\mu\text{g/mL}$ . Based on these observations, diaryl methane **6** and arakylaminopyridine **7** (Fig. 12) were selected for further testing against a larger panel of picornaviruses and their *in vivo* antiviral efficacy. Both compounds exhibited similar *in vitro* activity, inhibiting 12–15 of the 23 picornaviruses tested at concentrations less than 5  $\mu\text{g/mL}$ . In addition, the arakylaminopyridine was found to be more active *in vivo* in protecting cox A21-infected mice at a single oral dose of 37.5 mg/kg or at a continuous oral dose of 18.8 mg/mL per day. With these observed *in vitro* potency and *in vivo* efficacy, their potential clinical application to the treatment of picornavirus infection diseases appears to be limited. As for 2-(4-pyridylaminomethyl)benzimidazole analogues,<sup>115</sup> their *in vitro* activities against polio 2, cox B4, HRV 14, and enterovirus 70 were tested, showing that these analogues are particularly effective against enterovirus 70 with  $IC_{50}$  values as low as 0.52  $\mu\text{g/mL}$ . Cytotoxicity evaluation using



**Figure 11.** Structures of Synphar Laboratories, Inc., Edmonton, Canada.



**Figure 12.** Structures of diaryl methanes and arakylaminopyridines.

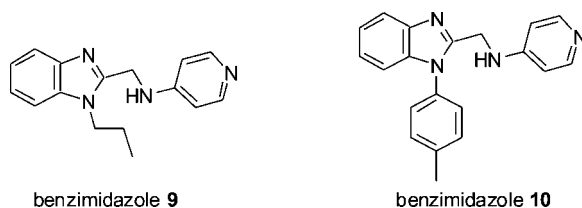
LLC-MK<sub>2</sub> and HaLa cells indicated that this series of compounds is not toxic up to concentrations more than 100 µg/mL (CC<sub>50</sub>). Representative compounds **9** and **10** (Fig. 13) prevented the development of Cox B4-induced hypoglycemia in mice for 2–4 days after infection when given intraperitoneally (i.p.) with an initial dose of 40 mg/kg/day followed by 80 mg/kg/day for 3 days. Results of clinical application are not available at the present time.

Some N- and O-substituted amino acid analogues were synthesized during the development of antiviral agents. Several substituted glycine analogues, as exemplified in Figure 14, showed moderate activity against cox A13, B4, and echo 11 with IC<sub>50</sub> values around 1.8 µg/mL. No *in vivo* studies were reported.

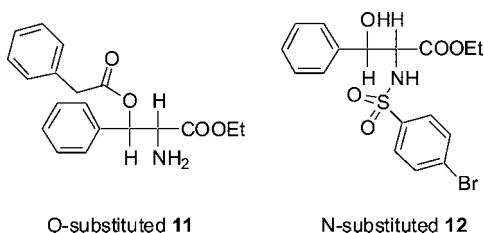
In summary, the active capsid-binding compounds generally are characterized by a long linear methylene spacer with either an aromatic or heterocyclic ring attached to both ends, making the molecules considerably hydrophobic. From genetic point of view, these common structural features implies that the structure of “sock-like” VP1 coat protein are highly conserved through the evolution of picornaviruses, with the intrinsic hydrophobic property of the VP1 pocket as well as the cavity shape well preserved. Therapeutically useful antiviral drugs should have broad antiviral spectrum, high potency, and low cytotoxicity. Therefore, molecules acting at the viral VP1 pocket, where only a subtle difference in the size is observed for various serotypes in the same genus or even for different genera, could be promising therapeutic agents for treating picornaviral infection.

### B. 3C Protease Inhibitors

To date, numerous compounds with significant *in vitro* activity against HRV and EV have been found. However, the majority of these compounds, as shown in the previous section, bind to the viral capsid and inhibit either viral attachment/adsorption or subsequent uncoating. In addition to agents that interfere with the early stage in the picornaviral life cycle, attempts have been made recently to develop inhibitors to block the virus-coded 2A or 3C proteases at the synthetic stage of the virus replication. Antipicornaviral agents designed to target the 3C protease, which is highly conserved among different viral serotypes, have exhibited great potential in therapeutic utility.



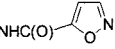
**Figure 13.** 2-(4-Pyridylaminomethyl)-benzimidazole derivatives.



**Figure 14.** Structures of amino acid analogues.

Since peptide aldehydes have been successfully used as inhibitors for cysteine and serine proteases and were shown to form reversible covalent adduct, the modified tripeptide aldehydes were designed and synthesized as inhibitors for HRV 3CP.<sup>117</sup> Molecular models based on the apo crystal structure of HRV-14 3CP and other trypsin-like serine proteases were constructed to approximate the binding of peptide substrate, generating transition state models of P<sub>1</sub>-P<sub>1</sub>' amide cleavage. Since glutaminal derivatives exist predominantly in the cyclic hemiaminal form, several isosteric replacements for P<sub>1</sub> carboxamide side chain were designed and incorporated into the tripeptide aldehydes. The synthesized compounds were found to be potent inhibitors of purified HRV-14 3CP with *K<sub>i</sub>*s ranging from 0.005 to 0.65 μM. As shown in Table III, these compounds have low

**Table III.** Biological Properties of Tripeptide Aldehydes Against HRV-14

R	<i>K<sub>i</sub></i> (μM) <sup>a</sup>	EC <sub>50</sub> (μM) <sup>b,c</sup>	TC <sub>50</sub> (μM) <sup>b,d</sup>	TI <sup>b,e</sup>
CH <sub>2</sub> CONH <sub>2</sub>	3.6	66	398	6
NHCHO	0.073	1.6	206	129
NHCOCH <sub>3</sub>	0.006	2.4	316	132
NHCOCF <sub>3</sub>	0.146	1.8	25	14
NHCOPh	0.012	1.0	51.2	51
	0.005	1.5	59.5	40
NHCO <sub>2</sub> CH <sub>3</sub>	0.132	5.0	56.2	11
NHCO <sub>2</sub> C(CH <sub>3</sub> ) <sub>3</sub>	0.066	2.0	56.2	28
NHSO <sub>2</sub> CH <sub>3</sub>	0.64	20	63	3
NHCON(CH <sub>3</sub> ) <sub>2</sub>	0.010	2.1	99	47
CH <sub>2</sub> CON(CH <sub>3</sub> ) <sub>2</sub>	0.005	1.3	63	48

The material in this table is taken part from Ref.117.

<sup>a</sup>*K<sub>i</sub>* data was measured against HRV-14 3CP. Standard deviation = ± %.

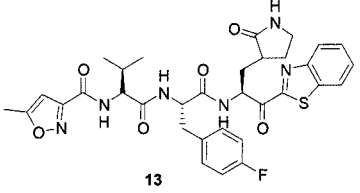
<sup>b</sup>HRV-14 infected H1-HeLa cell protection assay.

<sup>c</sup>50% effective concentration.

<sup>d</sup>50% toxic concentration.

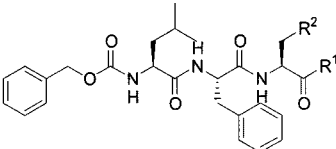
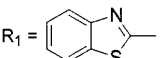

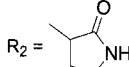
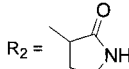
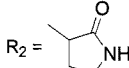
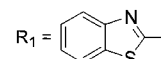

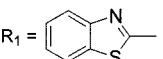
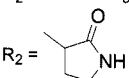
<sup>e</sup>Therapeutic index.

**Table IV.** Inhibitory, Anti-HRV Activity, and Cytotoxicity of Compound 13

 13	HRV-14	HRV-1A	HRV-10
	$K_i$ ( $\mu\text{M}$ )	0.0045	ND
$EC_{50}$ ( $\mu\text{M}$ )	0.34	0.34	0.25
$CC_{50}$ ( $\mu\text{M}$ )	250	250	250

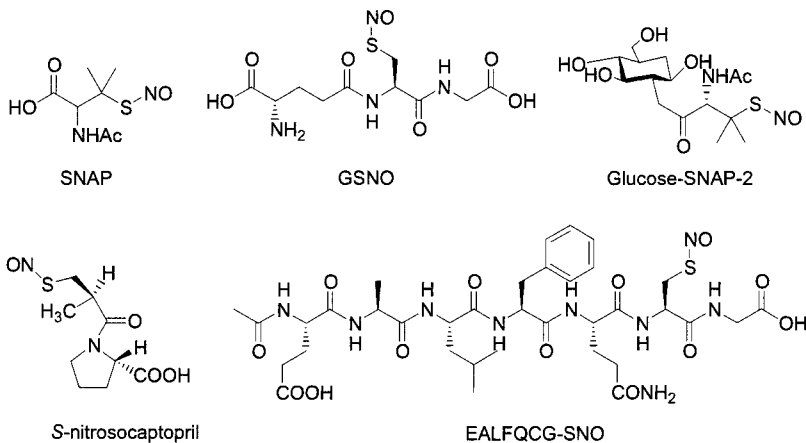
The material in this table is taken part from Ref.118.

**Table V.** Inhibitory, Anti-HRV Activity, and Cytotoxicity of Compounds 14–22

			
14	$R_1 = $  $R_2 = \text{NHCOCH}_3$	19	$R_1 = $  $R_2 = $ 
15	$R_1 = \text{HC}\equiv$ $R_2 = \text{NHCOCH}_3$	20	$R_1 = 2\text{-pyridine}$ $R_2 = $ 
16	$R_1 = \text{MeOH}_2\text{CC}\equiv$ $R_2 = \text{NHCOCH}_3$	21	$R_1 = \text{CH}_3$ $R_2 = $ 
17	$R_1 = \text{CH}_2\text{OCH}_3$ $R_2 = \text{NHCOCH}_3$	22	$R_1 = $  $R_2 = $ 
18	$R_1 = $  $R_2 = $ 		

Compd No.	Virus serotype	$K_i$ ( $\mu\text{M}$ )	$EC_{50}$ ( $\mu\text{M}$ )	$CC_{50}$ ( $\mu\text{M}$ )
14	HRV-14	1.7	> 25	25
14	HRV-16	0.31	> 25	ND
14	HRV-2	0.90	ND	ND
14	HRV-89	0.48	ND	ND
15	HRV-14	0.065	> 100	> 100
15	HRV-16	0.322	ND	ND
15	HRV-2	0.124	ND	ND
15	HRV-89	0.76	> 100	> 100
16	HRV-14	0.098	> 100	> 100
17	HRV-14	0.075	> 100	> 100
18	HRV-14	0.065	3.2	> 320
19	HRV-14	0.70	7.9	240
20	HRV-14	0.17	4.0	200
21	HRV-14	0.65	> 10	> 10
22	HRV-14	0.0045	0.335	251

The material in this table is taken part from Ref.119.

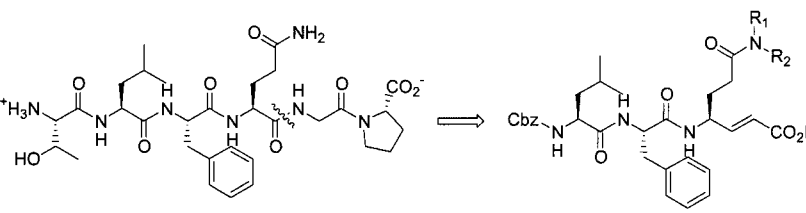
**Table VI.** Kinetic Parameters for the Inactivation of HRV-14 3C Protease by *S*-Nitrosothiols


The figure shows five chemical structures: SNAP (N-(3-(3-dimethylbutyl)thio)propyl)acetamide; GSNO (N-(3-(3-dimethylbutyl)thio)propyl)glycine; Glucose-SNAP-2 (a glucose derivative with a SNAP group at the 2-position); S-nitrosocaptopril (a proline derivative with a nitrosothio group); and EALFQCG-SNO (a heptapeptide with a nitrosothio group at the C-terminus).

Inhibitor	$k_{\text{inact}}$ ( $\text{min}^{-1}$ )	$K_i$ ( $\mu\text{M}$ )	$k_{\text{inact}}/K_i$ ( $\text{M}^{-1} \text{min}^{-1}$ )
SNAP	0.243	1.86	131
GSNO	0.237	0.36	656
Glucose-SNAP-2	0.982	5.04	195
<i>S</i> -NO Captopril	0.457	2.11	217
EALFQCG-SNO	0.107	0.02	5360

The material in this table is taken part from Ref.120.

micromolar antiviral activity, low toxicity, and reasonable therapeutic index. Along this line, structure-based design of ketone-containing tripeptidyl HRV 3CP reversible inhibitors were also reported.<sup>118</sup> An excellent example of such compounds (e.g., **13**) displayed potent 3CP inhibition activity and *in vitro* antiviral property when tested against HRV serotype-14 (see Table IV).

**Table VII.** Design of HRV 3CP Inhibitors


The reaction scheme shows the synthesis of HRV 3CP inhibitors. The starting material is a complex peptide derivative with a proline ring and a carboxylate group. It reacts with a carbonyl compound containing an amine group (R1-NH-CO-R2) to form a new inhibitor structure where the proline ring is substituted with the R1-NH-CO-R2 group. The resulting inhibitor has a Cbz group, a proline ring, and a carboxylate group (CO2Et).

R <sub>1</sub>	R <sub>2</sub>	serotype	EC <sub>50</sub> ( $\mu\text{M}$ )	CC <sub>50</sub> ( $\mu\text{M}$ )
H	H	14	0.54	> 320
CH <sub>3</sub>	H	14	5.6	> 100
CH <sub>3</sub>	CH <sub>3</sub>	14	4.0	> 100

The material in this table is taken part from Ref.121.





**Table X.** 5-Substituted Benzamides

R <sub>1</sub>	K <sub>obs</sub> /[I] (M <sup>-1</sup> S <sup>-1</sup> )	EC <sub>50</sub> (μM)	CC <sub>50</sub> (μM)
OH	54	> 100	> 100
	568	10.0	> 100
	286	5.6	> 100
	269	2.5	> 100
	96	5.8	> 100
	130	1.8	> 100
	163	1.0	> 100
	139	0.6	79

The material in this table is taken part from Ref. 127.

time- and concentration-dependent manner with second-order rate constants ( $K_{\text{inact}}/K_1$ ) ranging from 131 to 5,360/M/min (Table VI). The inactivated enzyme was shown to be reactivated by DDT, GSH, and ascorbate, indicating that the inactivation process was through an *S*-transnitrosylation process.

A new class of 3CP inhibitors containing a tripeptide binding determinant as well as a Micheal acceptor moiety capable of binding irreversibly to the active site cysteine of 3C enzyme was described as agents against rhinovirus (Table VII).<sup>121</sup> Indeed, analysis of the HRV-2 3CP X-ray crystal structure<sup>122</sup> revealed that only the *trans* P<sub>1</sub> Gln amide hydrogen atom interacted with the protease while the *cis* NH was found to be exposed to the solvent.

The P<sub>1</sub>-lactam-containing inhibitors (e.g., **24**) display enhanced 3CP inhibition activity along with improved antirhinoviral properties relative to the corresponding glutamine-derived molecules (e.g., **23**) (Table VIII). Being one of the most potent inhibitors in this class, compound **24** (AG-7088), which is formulated for intranasal delivery in Phase II trial, exhibited better potency and a broader spectrum of antirhinoviral activity than pleconaril towards clinical HRV isolates.<sup>123,124</sup> The median EC<sub>50</sub> value determined by microscopic CPE inhibition was slightly better for AG-7088 compared to

**Table XI.** Inhibitory Property of 1,5-Disubstituted Isatins Against HRV-14 3CP

R <sub>1</sub>	R <sub>2</sub>	HRV-14 K <sub>i</sub> (μM)
CH <sub>3</sub>	CO <sub>2</sub> H	10.0 ± 3.0
CH <sub>3</sub>	CONH <sub>2</sub>	0.051 ± 0.006
CH <sub>3</sub>	CONHCH <sub>3</sub>	12.0 ± 2.0
CH <sub>3</sub>	CON(CH <sub>3</sub> ) <sub>2</sub>	33.0 ± 7.0
( <i>E</i> )-CH <sub>2</sub> CH=CHPh	CONH <sub>2</sub>	0.011 ± 0.002
(CH <sub>2</sub> ) <sub>3</sub> Ph	CONH <sub>2</sub>	0.027 ± 0.005
CH <sub>2</sub> -β-naphthyl	CONH <sub>2</sub>	0.004 ± 0.003
CH <sub>2</sub> -2-benzo[ <i>b</i> ]thiophene	CONH <sub>2</sub>	0.002 ± 0.002
CH <sub>2</sub> (4-Me-C <sub>6</sub> H <sub>4</sub> )	CONH <sub>2</sub>	0.010 ± 0.002
CH <sub>2</sub> (3,4-di-MeC <sub>6</sub> H <sub>3</sub> )	CONH <sub>2</sub>	0.006 ± 0.002
CH <sub>2</sub> (3-OMe-β-naphthyl)	CONH <sub>2</sub>	0.029 ± 0.009
CH <sub>2</sub> (3,5-di-OMeC <sub>6</sub> H <sub>3</sub> )	CONH <sub>2</sub>	0.025 ± 0.004
CH <sub>2</sub> (6-OMe-β-naphthyl)	CONH <sub>2</sub>	0.003 ± 0.001
CH <sub>2</sub> (3-OHC <sub>6</sub> H <sub>4</sub> )	CONH <sub>2</sub>	0.011 ± 0.003

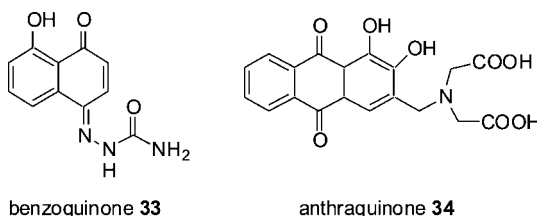
The material in this table is taken part from Ref. 128.

pleconaril ( $P = 0.02$ ) but was indistinguishable by spectrophotometric assay ( $P = 0.15$ ). In the case of clinical HRV isolates, however, the median EC<sub>50</sub> value determined for AG-7088 either microscopically or spectrophotometrically was < 1.0 μg/mL and was found to be > 10.0 μg/mL for pleconaril.

Symptom severity in patients with HRV-induced respiratory illness is correlated with elevated levels of inflammatory cytokines interleukin-6 (IL-6) and IL-8. AG-7088 was tested for its antiviral activity and ability to inhibit the production of IL-6 and IL-8 in a human bronchial epithelial cell line, BEAS-2B.<sup>125</sup> Infection of BEAS-2B cells with HRV-14 resulted in the production of both infectious virus and the cytokines IL-6 and IL-8. Treatment of HRV-14 infected cells with AG-7088 resulted in a dose-dependent reduction in the levels of infectious virus as well as a reduced IL-6 and IL-8 level in the cell supernatant. AG-7088 was able to inhibit the replication of the virus in BEAS-2B cells.<sup>126</sup>

In order to have more favorable pharmacokinetic properties and to develop orally available 3CP inhibitors, certain substituted benzamides as non-peptide inhibitors of HRV 3CP were invented.<sup>127</sup> α, β-Unsaturated keto benzamides (Table IX) showed good inhibitory property and moderate activity; yet 5-substituted benzamides (Table X) were found to be more active.

Evaluation of reversible, non-specific inhibitors of HRV 3C protease led to a novel series of 2,3-dioxindoles (isatins) by using a combination of protein structure-based drug design, molecular modeling, and structure–activity relationship analysis.<sup>128</sup> The C-2 carbonyl of isatin was envisioned to react in the active site of HRV 3CP with the cysteine responsible for catalytic proteolysis. Molecular modeling using the apo crystal structure of HRV-14 3CP and a peptide substrate model provided the design template for building recognition features into P<sub>1</sub> and P<sub>2</sub> subsites, respectively, from 5- to



**Figure 15.** Structures of quinone analogues.

1-positions of isatin. The synthesized compounds (see Table XI) were found to possess excellent inhibitory properties toward HRV-14 3CP compared to other proteolytic enzymes, including chymotrypsin and cathepsin B.

Recently, it was claimed that compounds having quinone moiety as well as quinone analogues are useful inhibitors for cysteine proteases, in particular, caspases and 3C cysteine proteases.<sup>129</sup> These compounds, as exemplified in Figure 15, have been tested against HRVs 1A, 1B, and 14 and show moderate *in vitro* activity with IC<sub>50</sub> values around submicro to micromolar range. Mechanistically, they are assumed to act as active Michael acceptors which are prone to attack by the cysteine residue and thus disrupt the ability of cysteine protease to cleave a peptide chain.

In summary, viral proteases play an essential role in the life cycle of many viruses such as picornaviruses, herpesviruses, retroviruses, and coronaviruses, and therefore, have been selected as targets for developing antiviral drugs. Protease inhibitors including saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir are available for treating diseases caused by HIV, a species of retroviruses. Antipicornavirus compounds targeting 3C protease are currently under active investigation. Among them, AG-7088, a potential treatment against rhinovirus causing the common cold, is now in Phase II clinical trial. Most recently, AG-7088 has shown to exhibit moderate *in vitro* activity against the coronavirus responsible for severe acute respiratory syndrome (SARS). Modeling studies<sup>130</sup> also indicate that AG-7088 is a promising starting point in the search for a treatment for SARS via targeting 3CL protease, the main protease controlling the coronavirus replication.

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