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Development of antiviral agents for enteroviruses

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Enteroviruses (EVs) are common human pathogens that are associated with numerous disease symptoms in many organ systems of the body. Although EV infections commonly cause mild or non-symptomatic illness, some of them are associated with severe diseases such as CNS complications. The current absence of effective vaccines for most viral infection and no available antiviral drugs for the treatment of EVs highlight the urgency and significance of developing antiviral agents. Several key steps in the viral life cycle are potential targets for blocking viral replication. This article reviews recent studies of antiviral developments for EVs based on various molecular targets that interrupt viral attachment, viral translation, polyprotein processing and RNA replication.

Keywords: capsid proteins, viral proteases, viral RNA replication, 5′ untranslated region

Introduction

Enteroviruses (EVs) are a common cause of infections in humans, especially children. They comprise over 70 distinct serotypes within the *Picornaviridae*. The subgroups of EVs contain the poliovirus (PV), coxsackie virus groups A and B (CVA and CVB), echoviruses and the numbered EVs such as EV71. Human EVs cause various diseases such as hand, foot and mouth disease (HFMD), herpangina, haemorrhagic conjunctivitis, meningitis, encephalitis, myocarditis, pleurodynia, paralysis and neonatal sepsis. Efforts towards global eradication of PV have succeeded in restricting its endemic presence to just four countries (Pakistan, India, Nigeria and Afghanistan) and a global case count of 1088 for 2007–08.[1] The non-polio EVs have continuously posed a threat to children, and annual outbreaks have been frequently reported. For example, EV71 is one of the most pathogenic EV serotypes that are currently circulating in the Asia-Pacific region. In 1997 and 1998, deaths associated with HFMD outbreaks due to EV71 infection were reported in Malaysia and Taiwan, respectively.[2] Several EV71 outbreaks with severe neurological complications have also subsequently been recorded in Korea, Japan, Malaysia, Vietnam and Australia. Most recently, in 2008, EV71 caused a total of 4496 cases, including 22 deaths, of HFMD in mainland China.[3] Other EVs such as echovirus 6, 9 and 30 and CVB5 were the most common causes of aseptic meningitis in children.[4] EV70 and CVA24 were associated with acute haemorrhagic conjunctivitis.[4] Additionally, coxsackie viruses were aetiologically linked with acute myocarditis.[4] To date, poliomyelitis can be controlled effectively by the administration of the polio vaccine. However, no powerful prophylaxis of non-polio EV infection is available. The early diagnosis of EV infection followed by antiviral therapies may prevent patients suffering from severe complications. Therefore, a need to develop potent antiviral agents for treating EV infection exists. The viral replication cycle of EVs involves a number of critical steps including virus adsorption, uncoating, protein translation, polyprotein cleavage, viral RNA replication and virus assembly, which are promising targets for drug discovery. This article summarizes recent advances in antiviral developments against EVs based on various molecular targets.

Antiviral agents that target viral capsid proteins

Numerous synthetic compounds exhibit *in vitro* antiviral activity by binding in a hydrophobic pocket beneath the canyon floor in the centre of the viral protein 1 (VP1), preventing viral attachment or uncoating. Pleconaril, one of the WIN compounds (a

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class of anti-EV and anti-rhinovirus compounds targeting the event of uncoating during viral replication), is a successful clinical candidate—it acts as a small-molecule inhibitor of EVs and rhinoviruses. It was developed to treat diseases associated with picornavirus infections.[5] In a virus-induced cytopathic effect assay, pleconaril was found to act against not only prototypic EV strains but also 215 clinical isolates that represent the most commonly isolated EV serotypes.[6] Pleconaril inhibited the 50% replication of all clinical isolates at a concentration of ≤ 0.03 μM.[6] The results of a Phase III clinical trial that involved nearly 2100 participants showed that treatment with pleconaril reduces the duration and severity of a cold in picornavirus-infected patients.[5] However, in 2002, the FDA did not approve pleconaril for the development of a common cold treatment because of concerns about the drug's safety. In 2003, pleconaril was licensed to Schering-Plough by Viropharma. In 2007, a Phase II clinical trial to investigate the effects of pleconaril nasal spray on common cold symptoms and asthma exacerbations following exposure to rhinovirus was completed.[7]

Although pleconaril was able to neutralize the cytopathic effect induced by many picornaviruses, it cannot inhibit EV71. A novel series of pyridyl imidazolidinones was then developed using computer-assisted drug design based on the skeletons of WIN compounds and their related molecule, pleconaril, as templates.[8] These imidazolidinone derivatives exhibited potent antiviral activity against several EVs, especially EV71. BPR0Z-194, 1-[5(40-bromophenoxy)pentyl]-3-(4-pyridyl)-2-imidazolidinone, a pyridyl imidazolidinone, effectively inhibited the activity of EV71.[9] The VP1 protein of EV71 has been identified as the molecular target for BPR0Z-194 using genetic approaches.[9] A single change in amino acid at position 192 of VP1 from Val to Met can render viruses resistant to BPR0Z-194. Structure–activity relationship studies have revealed that the chain length of the alkyl linker and the alkyl substituent at the oxime ether group influence the anti-EV71 activity of these antiviral agents.[10] More specific molecular interactions between VP1 protein and pyridyl imidazolidinones were investigated, and the results suggest that the ability to fit into the hydrophobic pocket and hydrophobic forces are key factors in determining the drug efficacy.[11]

Pirodavir (R77975) belongs to a series of pyridazine analogues. It is also a capsid-binding agent with potent activity against 16 EV serotypes and rhinovirus groups A and B.[12] Intranasal sprays of pirodavir reduced viral shedding, but offered no clinical benefit to individuals with rhinovirus-associated symptoms.[13] BTA-798, the oxime ether analogue of pirodavir, was synthesized at Biota Company and was developed to treat human rhinovirus (HRV) infections. A Phase I clinical trial of the use of BTA-798 to treat HRV infections in high-risk chronic obstructive pulmonary disease and asthma patients has been completed, and a Phase II trial with this compound is planned to begin in 2008.[7]

Viral proteases as targets for antiviral drug developments

Proteins 2A and 3C are proteases of picornaviruses and are important for viral polyprotein processing. As 2A protease is highly conserved in rhinoviruses and EVs, and 3C is present in a unique folding structure that differs from that of known cellular proteases, these viral proteases are potential targets for antiviral agent design. Several compounds that target 2A protease have been identified and developed into antiviral drugs. Alkylating agents, such as iodoacetamide and *N*-ethylmaleimide, have been proven to reduce 2A activity.[14] Caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD.fmk) and benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (zIETD.fmk) can also block HRV and CVB4 2A activity *in vitro* and *in vivo*.[15] zVAD and zIETD have been demonstrated to reduce HRV multiplication in cell culture. As the substrate-binding pocket of elastase is similar to that of 2A, two substrate-derived elastase inhibitors, elastatinal and methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone, have been reported to inhibit the *in vitro* proteolytic activity of 2A and reduce the viral yields of HRV-14 and PV type 1.[16]

Several antiviral compounds that target 3C protease have been developed based on the mimicking of 3C substrates. Many of these compounds are peptides that contain three to five amino acids and an aldehyde group, which serves as an electrophilic anchoring group.[7] Some modifications have been made in developed peptidic 3C inhibitors to enhance their antiviral activity. The replacement of a scissile amide carbonyl with a Michael acceptor, an electron-withdrawing group, can cause the formation of stable irreversible covalent bonds between the 3C protease and peptidic 3C inhibitors.[17] Compound AG7088 (rupintrivir) is the most successful antiviral agent of these modified peptidic 3C inhibitors.[18] This compound also exhibits great antiviral activity against picornaviruses including HRV, CVA21, CVB3, echovirus 11 and EV70.[19] Although successful trials in patients who were infected experimentally with HRV have been reported, AG7088 cannot reduce virus titre or moderate disease severity in naturally infected HRV patients, and so the clinical development has been terminated.[7] Based on the knowledge gained from studies on peptidic 3C inhibitors, non-peptidic 3C inhibitors derived from the co-crystal structure of substrates bound to 3C have been developed.[20] Another example is one 3C inhibitor with 2,3-dioxindole (isatin), which has a high affinity for the protease pocket.[21] Some screening assays have also been developed to screen potential 3C inhibitors. A cell-based EV71 3C protease assay was established recently, as a potential method for screening more 3C protease inhibitors for picornaviruses.[22]

Antiviral agents targeting viral proteins involved in viral RNA replication machinery

The replication of enteroviral RNA occurs within the replication complex, which is associated with the membrane of virus-induced vesicles in the cytoplasm of infected cells. The viral RNA replication complex comprises a variety of viral proteins including 2B, 2C, 2BC, 3A, 3B (VPg), 3AB, 3CD and 3D. Some of them have been studied as targets for developing antiviral agents and are discussed below.

2C protein

The 2C protein of picornavirus contains three conserved motifs (motifs A, B and C) that are typically found in NTP-binding proteins or in members of helicase superfamily III. The ATPase and GTPase activities of PV 2C protein have been

demonstrated.[23] However, many studies have failed to demonstrate the *in vitro* RNA helicase activity of the 2C protein. Several anti-enteroviral inhibitors have been found to target 2C protein based on genetic evidence. One benzimidazole derivative, 2-(alpha-hydroxybenzyl)-benzimidazole (HBB), has been shown to exhibit selective anti-picornaviral activity.[24] Resistance to HBB of echovirus 9 has been demonstrated to be due to mutations in the 2C protein.[24] The synergistic effect of HBB and guanidine on the production of picornavirus has been observed, indicating different inhibitory mechanisms of both compounds.[24] Another benzimidazole derivative, 1-(4-fluorophenyl)-2-[(4-imino-1,4-dihydropyridin-1-yl)methyl] benzimidazole hydrochloride (MRL-1237), exhibited antiviral activity against PV and CVB.[25] CVB was more sensitive to MRL-1237 than to PV. The guanidine-resistant mutants were cross-resistant to MRL-1237, indicating that both of them may have the same target.[25] Recently, a newly synthesized benzimidazole derivative, 1-(2,6-difluorophenyl)-6-trifluoromethyl-1*H*,3*H*-thiazolo[3,4-a]benzimidazole (TBZE-029), has been identified to exhibit activities against various EVs, and it is reported that this compound targets the picornaviral 2C region.[26] TBZE-029-resistant virus exhibited cross-resistance with other 2C inhibitors such as guanidine, HBB and MRL-1237.

3A protein

Protein 3A is thought to serve as a scaffold of the viral RNA replication complex and is highly conserved in both EVs and rhinoviruses. A benzimidazole derivative, enviroxime [2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketone oxime], reportedly exhibits potent activity against EVs *in vitro*.[27] The 50% viral cytopathogenic effect inhibitory dose of enviroxime against 11 EV70 and 15 CVA24 variant isolates ranged from 0.01 to 0.65 mg/L.[27] Enviroxime exhibits activity against rhinovirus in humans based on a series of rhinovirus challenge studies, although it has emetic side effects and poor oral bioavailability.[28] A series of vinylacetylene and C2 analogues of enviroxime have been synthesized to improve oral bioavailability and pharmacological profile.[29,30] Among vinylacetylene analogues, one analogue (compound **12**) has been shown to be efficacious after being orally administered in the treatment of CVA21-infected mice.[29] A C2 analogue of enviroxime with a primary amino substitution has been found to be the most active compound against rhinoviruses 2, 14 and 16, PV1 and CVA21.[30]

3D polymerase

One promising antiviral therapy involves nucleoside analogues that enhance the mutation frequency of RNA virus, resulting in an 'error catastrophe' and loss of viral viability. One such analogue, ribavirin, is incorporated into the viral genome causing lethal mutagenesis.[31] Ribavirin has been used in combination with interferon in the treatment of hepatitis C virus (HCV) infection and also can inhibit the replication of CVB3 and EV71 in animal models.[32,33] Valopicitabine (2′-*C*-methylcytidine), the oral prodrug of another nucleoside analogue, targets HCV polymerase and has been demonstrated to exhibit antiviral activity against all three PV strains with EC_{50} values of 3.9–29 μM.[34] Moreover, the adenosine analogue of valopictabine has been shown to exhibit equipotent activity against all three PV strains (~5 μM).[34] A series of 5-substituted cytidine analogues have been synthesized and exhibit greater antiviral activity against PV and CVB3 than does ribavirin.[35] This 5-nitrocytidine inhibits the activity of PV RNA-dependent RNA polymerase, and this inhibition may be the cause of the loss of viral viability.[35] Recently, a novel series of N-6-substitiuted purine analogues with the ability to reduce the titre of PV and CVB3 was synthesized.[36] Besides nucleotide analogues, other inhibitors that target viral RNA-dependent RNA polymerase have also been identified. Amiloride, a cellular ion transport blocker, inhibited the RNA replication of CVB3.[37] Mutations in the viral RNA-dependent RNA polymerase conferred CVB3 resistance to the compound. This compound had a stronger antiviral effect against CVB3 than did HRV2. However, the mechanisms of antiviral activity differ significantly between these two viruses. Pyrazolo[3,4-*d*] pyrimidine was identified as a novel class of EV71 inhibitor with broad-spectrum activity against EVs.[38] Our data from analysing the sequences of pyrazolo[3,4-*d*]pyrimidine-resistant viruses suggest that the viral RNA polymerase of EV71 is the target of the drug (T.-C. C. and S.-R. S., unpublished data).

Antiviral agents that target viral RNA in the 5′ untranslated region (5′ UTR)

The 5′ UTR of EV is known to have important roles in viral translation and RNA synthesis. Some approaches targeting 5′ UTR have been found, such as antisense phosphorothioate DNA that has been used against CVB3.[39] Phosphorodiamidate morpholino oligomers (PMOs) are designed as single-stranded DNA-like antisense agents against RNA viruses through interfering with gene expression. EnteroX, a peptide PMO, designed to target the sequence in the internal ribosome entry site of 5′ UTR, which is highly conserved across human EVs and rhinoviruses, is active against not only HRV14 but also clinical isolates of CVB2 and PV1 in cell culture experiments.[40]

Conclusions

The design of new antiviral compounds that target specific steps in the viral replication cycle and the modification of existing antiviral compounds are the current approaches for developing antiviral drugs. However, the emergence of drug-resistant variants could occur due to high mutation rates of EVs. The use of drug combinations seems to be a way to delay or prevent the appearance of drug-resistant viruses. For example, 'cocktail therapy' extends the life of HIV-infected patients who suffer from infection with drug-resistant viruses. Many potent EV inhibitors, mentioned earlier, act on various targets in viral replication cycles. Some have been or are being tested in clinical trials. These compounds, used alone or in combination, may have the potential for the treatment of EV infection. To date, no powerful prophylaxis of non-polio EV infection is available. No antiviral agent has been approved by the FDA for treating EVs. The continued development of drugs for the treatment of enteroviral infection is essential. Moreover, many other potentially interesting targets are necessary to be further explored.

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Transparency declarations

None to declare.

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