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Review

Antiviral drug discovery for the treatment of enterovirus 71 infections

Luqing Shang, Mengying Xu, Zheng Yin *

College of Pharmacy & State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, PR China

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ABSTRACT

Enterovirus 71 (EV71) is a small, positive-sense, single-stranded RNA virus in the genus *Enterovirus*, family *Picornavirus*. It causes hand, foot and mouth disease in infants and children, which in a small percentage of cases progresses to central nervous system infection, ranging from aseptic meningitis to fatal encephalitis. Sporadic cases of EV71 infection occur throughout the world, but large epidemics have occurred recently in Southeast Asia and China. There are currently no approved vaccines or antiviral therapies for the prevention or treatment of EV71 infection. This paper reviews efforts to develop antiviral therapies against EV71.

Contents

* Corresponding author. Tel.: +86 22 23500963; fax: +86 22 23507760.
E-mail address: zheng_yin@nankai.edu.cn (Z. Yin).

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1. Introduction

Enterovirus 71 (EV71) is the causative agent of hand, foot and mouth disease (HFMD) in infants and children. HFMD usually presents as a mild febrile disease with a localized rash, but some patients develop infection of the central nervous system (CNS), with illness ranging from aseptic meningitis through fatal encephalitis. Sporadic cases of EV71 infection occur throughout the world, but large epidemics have principally been seen in Southeast Asian countries and in China. EV71 was initially isolated from an adult HFMD case in Wuhan city in China 1987 (Zheng et al., 1989). According to data from the Chinese Center for Disease Control and Prevention (CDC), HFMD was listed first among category C infectious diseases from 2009 to 2011, in terms of incidence and death rate. More than 1,600,000 cases of EV71 infection, with more than 500 deaths, were reported in China in 2011.

No vaccines or antiviral therapies are available for the prevention or treatment of EV71 infection, but progress has been made in recent years. This paper surveys current approaches for the discovery of novel, effective drugs against EV71.

2. Classification, genome and viral proteins

EV71 is a non-enveloped, positive-sense, single-stranded RNA virus in the genus *Enterovirus*, family *Picornaviridae*. Its ~7400-base genome contains a single long open reading frame (ORF) with untranslated regions (UTR) at the 5′ and 3′ ends and a variable length poly-A tail at the terminus of the 3′UTR (Fig. 1) (Lin et al., 2009a,b). The ORF is divided into three consecutive parts, P1, P2 and P3. The viral RNA encodes a large polyprotein which is cleaved by virus-encoded and host proteases to produce the mature proteins.

Processing of the P1 region yields the structural proteins, VP1–VP4. VP1, VP2 and VP3 are located at the surface of the viral capsid. They have an antiparallel β-barrel structure, of the "jelly roll" type; a depression within the β-barrel is believed to be the attachment site of the virus (Dimmock et al., 2001). Proteins VP1–3 form a wedge, which is assembled into virus particles, with the shorter VP4 located entirely inside the capsid. Processing of the P2 and P3 regions produces the nonstructural proteins 2A–2C and 3A–3D, respectively, which include the viral proteases and the RNA-dependent RNA polymerase (RdRp) (see below) (Brown and Pallansch, 1995).

3. Epidemiology

EV71 is divided into four genotypes, designated A, B, C and D (previously designated as C4), according to the alignment of the complete VP1 or VP4 sequence. Genotypes circulating in the past and present have recently been tabulated (Chan et al., 2010). During the past 20 years, major EV71-associated HFMD outbreaks have primarily been caused by genotype C viruses, sub-divided as C1–C3 and C5. From 1986 to 2008, subgenotype C1 was almost always detected in China, the United Kingdom, the USA, Thailand and Australia. Subgenotype C2 was isolated as major causative agent of Taiwan outbreak in 1998. The subgenotype C3 was distributed in China in 1997 and Korea in 1999, while subgenotype C5 caused hundreds of deaths in Taiwan, Vietnam and Thailand between 2001 and 2003.

Currently, genotype D, previously designated as subgenotype C4, circulates in the Asian region (Tan et al., 2011). Large-scale outbreaks of HFMD have emerged in mainland China since 2007. A nationwide epidemic occurred in 2008, beginning in Fuyang city, Anhui Province, and spreading quickly into other provinces, with more than 176,000 cases and at least 40 deaths (Zhang et al., 2010a,b). Evidence from both traditional and molecular epidemiology confirmed that recent HFMD outbreaks have been caused primarily by genotype D (Tan et al., 2011).

4. Disease manifestations

The most common form of EV71 infection is HFMD, typically seen in outbreaks in young children, which spreads principally through direct contact and the fecal-oral route. Initial signs and symptoms include fever, headache, sore throat and a flu-like syndrome. Within a few days, patients develop painful ulcerated lesions in the nose, mouth and throat, accompanied by a rash that typically affects the hands and feet. In addition to HFMD, EV71 infection may involve the upper respiratory tract, the gastrointestinal tract, the cardiovascular system and the central nervous system. Neurological diseases range in severity from aseptic meningitis to acute flaccid paralysis and fatal encephalitis.

Chang et al. (2004) classified symptomatic EV71 infection in 4 stages:

1. HFMD and herpangina.
2. Central nervous system involvement.
3. Cardiopulmonary involvement.
4. Long-term sequelae.

Most EV71 infections remain at stage 1, some progress to stage 2, and only a few advance further to the most severe condition, stage 3. Some survivors of stage 3 have long-term sequelae during convalescence (stage 4). Recent follow-up studies in children have demonstrated that EV71 infection can cause long-term neurological sequelae, including delayed development and reduced cognitive function (Chang et al., 2007).

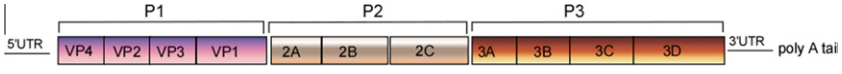


Fig. 1. Schematic of the enterovirus 71 genome structure. Adapted and modified from published literature (Brown and Pallansch, 1995).

5. Vaccines

With the success of live, attenuated and inactivated polio vaccines in preventing poliomyelitis and eradicating polioviruses from most countries of the world, EV71 is now considered a top candidate for new vaccine development. A number of different types of vaccines have been evaluated. Wu et al. (2001) tested the immunogenicity and protective efficacy of heat-inactivated virions, a VP1 DNA vaccine and the VP1 protein expressed in *Escherichia coli* in mice. All three candidate vaccines induced neutralizing antibodies, but only the inactivated whole-virus vaccine protected mice from fatal infection. EV71 virus-like particle (VLP)-based vaccines have been investigated by researchers in Taiwan. VLPs produced in a baculovirus expression system were highly immunogenic in mice and macaque monkeys (Chung et al., 2008; Lin et al., 2012).

The usefulness of VP1 to generate a subunit vaccine has recently been highlighted. Several candidates based on VP1, including synthetic peptides containing VP1 linear neutralizing epitopes (Foo et al., 2007), bacterially or virally expressed VP1 (Chiu et al., 2006), recombinant VP1 produced in *E. coli* (Liu et al., 2010) and VP1 DNA vaccine (Tung et al., 2007), are immunogenic and show good immune response in vaccinated mice. In another approach, Tan et al. prepared a candidate live, attenuated vaccine by mutating potential molecular determinants of neurovirulence that were identified in poliovirus type 1 (Tan and Cardosa, 2007). The genetically modified EV71 viruses were highly immunogenic when given to monkeys by the intravenous route, but caused CNS infections. They would therefore need further attenuation to be acceptable for clinical trials. Chang et al. (2012a,b) reported the development of a Vero cell-based, chemically inactivated EV71 vaccine candidate. The clinical isolate E59 was characterized, validated and selected as the vaccine strain. Formulated together with alum adjuvant, the vaccine was found to induce strong neutralizing antibody responses in rodents.

Several inactivated whole-virus vaccines have entered clinical trials (Chou et al., 2012). The first Phase I clinical trial of an EV71 vaccine for children was completed with encouraging results by Taiwan's National Health Research Institutes (NHRI) in 2011. The vaccine produced more than 600 times the normal level of antibodies in adults (Li et al., 2012). Recently, Meng et al. (2012) published data from a Phase 1 clinical trial of an inactivated EV71 vaccine in healthy Chinese adults and children. The vaccine showed acceptable tolerability and did not elicit anti-nuclear antibodies. Good immune response was observed with one dose. Additionally, Mao et al. (2012) compared the immunogenicity of different inactivated EV71 vaccines and suggested that the standardized antigen unit could potentially be used to evaluate antibody responses and protective efficacy in future clinical trials.

6. The EV71 replication cycle and specific inhibitors

6.1. Activity of P1 region-encoded viral proteins

6.1.1. Virion binding to cellular receptors

Analysis of the crystal structure of EV71 revealed that the genome is enclosed in an icosahedral particle consisting of protein units V1, VP2 and VP3, while VP4 is attached to the inner surface of protein shell (Plevka et al., 2012; Wang et al., 2012a,b). Among these capsid proteins, VP1 is believed to be the major contributor in EV71 pathogenesis, as it is exposed on the surface of the virion and is targeted by neutralizing antibodies (Ku et al., 2012; Lim et al., 2012). A specific VP1 mutation (Q145E) promoted viral binding and RNA accumulation, contributing to infectivity and mouse lethality (Huang et al., 2012).

VP1 is involved in the recognition of EV71 receptors on the surface of host cells and (Tan and Cardosa, 2007; Sivasamugham et al., 2006). Several cellular receptors have been identified. Human scavenger receptor class B, member 2 (SCARB2), also known as lysosomal integral membrane protein-II or CD36b-like-2, has been shown to directly and specifically bind EV71. Expression of human SCARB2 enables normally nonsusceptible cell lines to support virus propagation and develop cytopathic effects (Yamayoshi et al., 2009). EV71 binds to SCARB2 via a canyon of VP1 around residue Gln-172. Soluble SCARB2 converts EV71 virions from 160S to 135S particles, indicating that it is an uncoating receptor of the virus (Chen et al., 2012).

Yorihiro et al. (2009) have also identified human P-selectin glycoprotein ligand-1 (PSGL-1; CD162) as a functional cellular receptor for the entry and replication of EV71 in leukocytes. PSGL-1 is a sialomucin membrane protein which has a major role in the early stages of inflammation. The N-terminal region of PSGL-1 specifically binds to EV71 and facilitates its replication in non-leukocyte cell lines. PSGL-1 expression allowed EV71 entry and replication and the development of cytopathic effects in otherwise non-susceptible mouse L929 cells. However, an *in vivo* study using transgenic mice revealed that expression of human PSGL-1 failed to increase the infectivity of EV71. The result indicates that PSGL-1 alone may not be sufficient to modulate infection with EV71 (Li et al., 2012).

The two cellular and functional receptors of EV71 (SCARB2 and PSGL-1) are highly glycosylated membrane proteins. Lin et al. (2009a,b) postulated that the receptor for EV71 on immature dendritic cells is DC-SIGN, and demonstrated that human dendritic cells can be infected with EV71 via DC-SIGN. Recent studies demonstrated that cell surface sialylation is a key regulator that assists the attachment of EV71 to host cell (Su et al., 2012). Focusing on epithelial cells of the gastrointestinal tract, Yang et al. (2009) showed that terminal sialic acid-linked glycans are involved in EV71 infection of DLD-1 intestinal cells.

6.7.2. Inhibitors of receptor binding

A number of approaches have been published for the discovery of antivirals targeting EV71 host receptor binding. Infection is inhibited by antibodies against SCARB2 and soluble SCARB2 in a dose-dependent manner (Yamayoshi et al., 2009). Similarly, infection can be inhibited in a dose-dependent manner with an antibody against amino acids 42–61 of PSGL-1, as well as by competition with soluble PSGL-1 (Yorihiro et al., 2009). Yang et al. (2009) demonstrated that the removal of sialic acid residues from plasma membrane proteins by sialidase protects EV71-susceptible DLD-1 intestinal cells from infection. Another study revealed that EV71 infection of immature dendritic cells via DC-SIGN was reduced by up to 50% by treatment with anti-DC-SIGN antibody (Lin et al., 2009a,b).

6.7.3. Virus entry and uncoating

A hydrophobic pocket within the VP1 protein is believed to be the attachment site of EV71 (Hogle et al., 1985). Uncoating of the viral particle, with release of the RNA genome, requires a certain degree of capsid structure with enough vacant space for conformational changes and uncoating to take place.

6.7.4. Inhibitors of entry and uncoating

Binding of inhibitors in the VP1 pocket may induce a conformational change in the canyon floor and thus prevent adsorption of the viruses to the host cells (Paul et al., 2003). In addition, insertion of a compound into the VP1 hydrophobic pocket may lead to an increase in the stability of the viral particle, rendering the virus resistant to uncoating, a process necessary for the release of viral RNA.

A number of capsid binding molecules have been investigated as antiviral agents against EV71 (Fig. 2). The series of "Win" compounds, designed by the Sterling-Winthrop Pharmaceutical Company, are the most widely investigated chemical structures among these agents (Diana et al., 1985). With the Win compound skeleton as a template, a structure-based drug design group at the NHRI in Taiwan has generated a library of virtual compounds whose minimum-energy conformations bear close similarity to the shape of the VP1 pocket of human rhinoviruses, and may also fit into the cavity of EV71. These studies have resulted in the discovery of a series of imidazolidinone derivatives, such as BPR0Z-112 and -284, possessing potent activity against EV71 (IC_{50} = 0.35–0.04 μM) (Shia et al., 2002, 2003).

A series of publications by the same group later documented various chemical modifications with good inhibitory activities, such as BPR0Z-194 (IC_{50} = 1.55 μM). In comparison with BPR0Z-194, BPR0Z-103 with a chlorophenyl substitution on the phenoxyl ring enhanced potency about 10-fold (IC_{50} = 0.13 μM), and BPR0Z-299 with a trifluoromethyl oxadiazole substitution increased antiviral potency 70-fold more (IC_{50} = 0.021 μM) (Shih et al., 2004a,b,c; Chen et al., 2008). BPR0Z-101 containing an oxime ether on the phenoxyl ring dramatically increased the anti-EV71 activity of pyridyl imidazolidinone (IC_{50} = 0.0012 μM) (Chern et al., 2004), and the introduction of a methyl group at the central position of the linker between the imidazolidinone and the biphenyl groups (BPR0Z-033, IC_{50} = 0.0088 μM) resulted in markedly improved antiviral activity (Chang et al., 2005). BPR0Z-074, which was designed based on size and hydrophobic forces, was the most potent inhibitor, with an IC_{50} of 0.0008 μM. Time-course studies showed that imidazolidinones effectively inhibited the early stages of EV71 viral infection, suggesting that VP1 is highly likely to be the molecular target for these compounds. They are currently under active investigation to evaluate their therapeutic potential (Fig. 2).

Pleconaril, which possesses broad-spectrum activity against enteroviruses, was tested for its antiviral activity against EV71 (Pevear et al., 1999), but it failed to neutralize the cytopathic effect induced by an EV71 strain isolated from the 1998 outbreak in Taiwan (Shia et al., 2002). Interestingly, a recent study demonstrated that the survival of EV71-infected mice treated with pleconaril (80 mg/kg) at 2 h post-infection was higher than in the control group (Zhang et al., 2012). Further investigation is needed for evaluation of its potential as an anti-EV71 agent.

Pirodavir, discovered by the Janssen Research Foundation, possessed significant activity against picornavirus replication (Watson et al., 2003). Novel pyridazinyl oxime ethers derived from pirodavir, BTA39 and BTA188, significantly inhibited EV71 replication, with IC_{50} values of 0.001 μM and 0.082 μM, respectively, while pirodavir was a much less effective inhibitor of EV71, having a IC_{50} value of 5.42 μM (Barnard et al., 2004). Both compounds appeared to be orally bioavailable in animals (Rotbart, 2002) (Fig. 2).

BW683C, 4′,6-Dichloroflavan, with a flavanoid-like skeleton, was highly effective against some picornaviruses. In order to improve their potency and broaden their antiviral spectrum, synthetic flavanoids substituted with halo-, cyano- and amidino-groups were prepared and tested for *in vitro* activity against EV71 (Genovese et al., 1995; Prendergast, 2001; Conti et al., 1998). BW683C exhibited the most potent activity (IC_{50} = 0.45 μM). However, compared

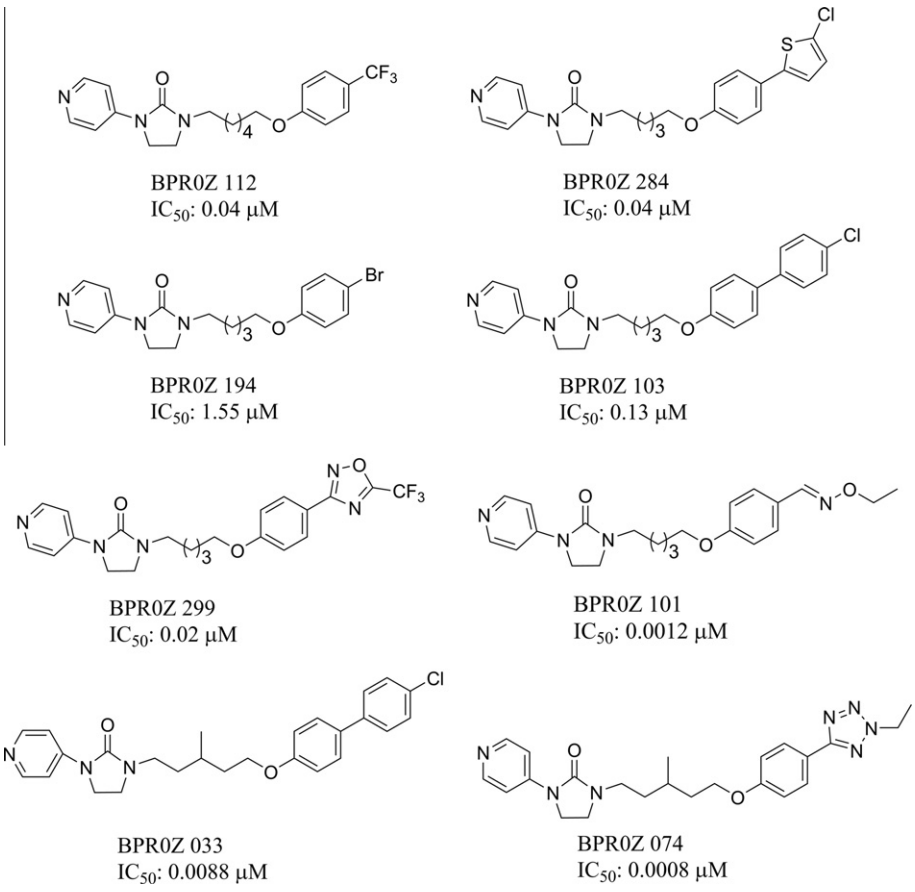


Fig. 2. Structure and biological activities of known capsid proteins inhibitors: BPR0Z-112, BPR0Z-284 (Shia et al., 2002, 2003); BPR0Z-194, BPR0Z-103, BPR0Z-299 (Chen et al., 2008); BPR0Z-101 (Chern et al., 2004); BPR0Z-033, BPR0Z-074 (Chang et al., 2005); Pleconaril (Pevear et al., 1999); Pirodavir (Watson et al., 2003); BTA39, BTA188 (Barnard et al., 2004); BW683C (Prendergast, 2001); NF449 (Arita et al., 2008).

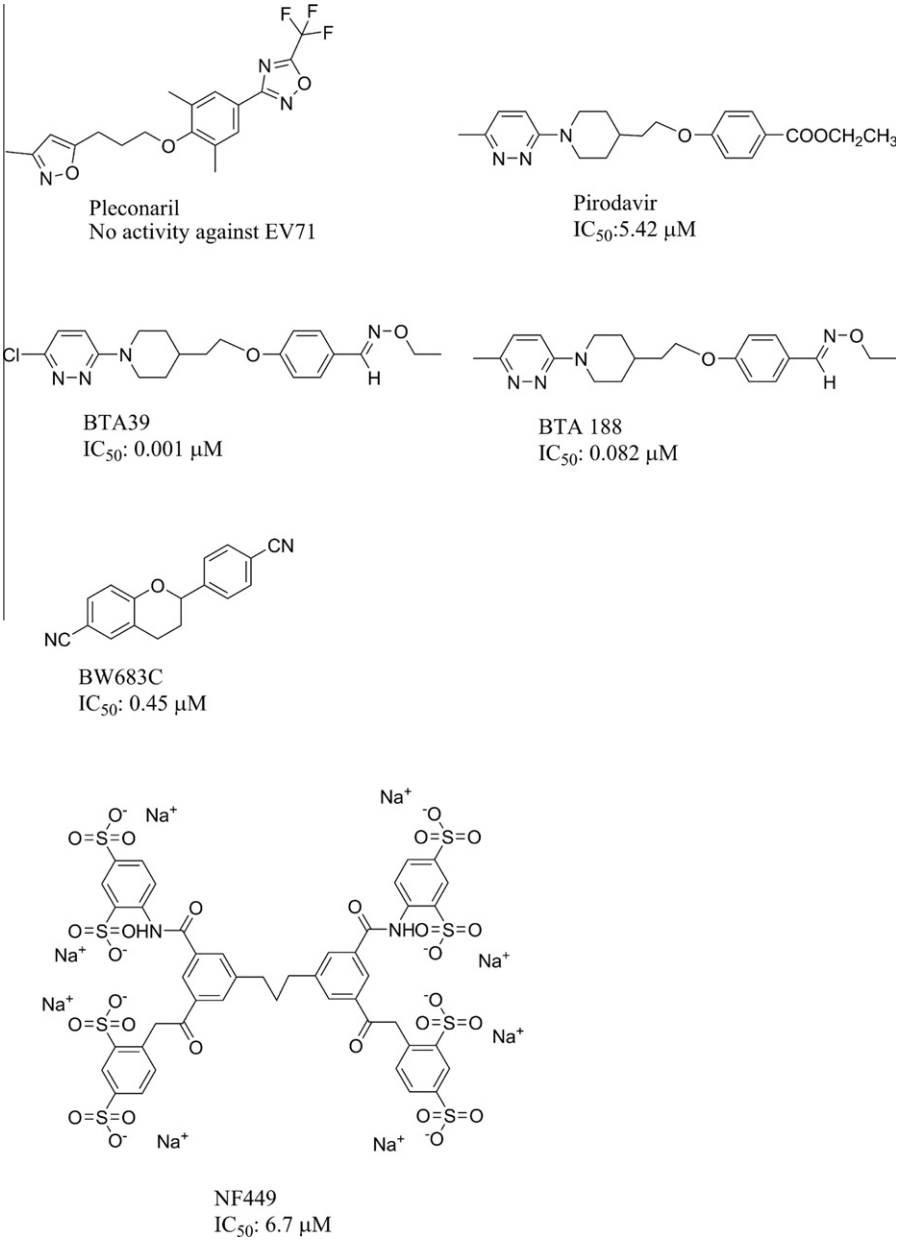


Fig. 2. (*continued*)

to the EV71-specific imidazolidinones discovered at NHRI (Taiwan), it is 10-fold less potent. The greater potency observed for the imidazolidinones could be attributed to their more efficient occupation of the VP1 pocket, to produce structurally more stable virions (Fig. 2).

Lactoferrin is an iron-binding glycoprotein that is present in the milk, saliva and other biological fluids of mammals, which has bacteriostatic and bacteriocidal activities (Arnold et al., 1977). The antiviral activity of bovine and human lactoferrin against EV71 was investigated. Bovine lactoferrin had a 10-fold stronger antiviral effect (IC_{50} = 10.5–24.5 μg/mL against 4 different EV71 strains) than human lactoferrin (IC_{50} = 103.3–185.0 μg/mL against 2 different strains) in plaque reduction assays (Weng et al., 2005). Lactoferrin may therefore be an effective and tolerable natural antiviral agent.

NF449 is an inhibitor identified by screening a pharmacologically active drug library, targeting poliovirus and EV71 pseudoviruses. It is a suramin analogue with antiviral activity against EV71 (IC_{50} = 6.7 μM with CC_{50} > 1000 μM) (Arita et al., 2008). A mode-of-action study demonstrated that NF449 blocked EV71 infection at the step of virus binding. The determinants of the resistance to NF449 were mapped to VP1 (Fig. 2).

Several recent reports have described the anti-EV71 activity of natural products and other compounds. The water extract of dried buds of *D. genkwa Sieb. et Zucc.* (DGFW) exhibited anti-EV71 activity without producing any cytotoxic effect. The mode of action study indicated that the extract inhibits viral replication by targeting viral entry. However, the active principle still remains to be elucidated (Chang et al., 2012a,b).

Sulfated polysaccharides from seaweed have broadly biological activities. Kappa carrageenan recently was reported to reduce plaque formation and prevent viral replication before or during viral absorption of EV71. The virus binding assay revealed that kappa carrageenan is able to bind EV71, forming carrageenan–virus complexes, whereby the virus–receptor interaction may be disrupted (Chiu et al., 2012).

A 15-mer synthetic peptide SP40 (Ac-QMMRKVELFTYMRFD-NH_2), spanning from position 118 to 132 in the VP1 region, was revealed to significantly reduce cytopathic effects of all EV71 strains. The mechanism studies found that the SP40 peptide was able to block viral attachment to host cells (Tan et al., 2012).

6.8. Activities of P2 region-encoded viral proteins

The initial step in the translation of the positive-stranded picornavirus genome is the formation of a single large polyprotein of about 2000 amino acids (~250 kDa). The polyprotein is rapidly processed into functional, mature viral proteins by co- and post-translational cleavages executed by the viral 2A and 3C proteases ($2A^{pro}$ and $3C^{pro}$) (Bazan et al., 1988; Racaniello et al., 2001; Ryan et al., 1997). These proteins appear to offer great opportunities for drug discovery.

6.8.1. The $2A^{pro}$ protein

Similar to poliovirus $2A^{pro}$, the EV71 polyprotein first undergoes an autocatalytic cleavage by $2A^{pro}$ to separate the capsid protein in the P1 region from the viral replication proteins in the P2-P3 region (Toyoda et al., 1986; Sommergruber et al., 1989; Hellen et al., 1992). The separation of the 3C and 3D proteins is also achieved by $2A^{pro}$. Expression of EV71 $2A^{pro}$ also leads to cleavage of the eukaryotic initiation factor 4GI (eIF4GI) (Kempf and Barton, 2008) and the poly-A binding protein (PABP) (Joachims et al., 1999). Cleavage of eIF4GI by $2A^{pro}$ inhibits cap-dependent translation of cellular mRNAs, without affecting the translation of viral RNA (Kuechler et al., 2002). Several genetic studies have shown that $2A^{pro}$ may have an essential role in viral RNA replication; however, the mechanism is not well understood (Li et al., 2001; Barton et al., 2002).

6.8.2. Drugs targeting $2A^{pro}$

Recently, the 6-amino acid peptide LVLQTM was demonstrated to bind the $2A^{pro}$ active site and significantly inhibited eIF4GI cleavage by $2A^{pro}$ (Falah et al., 2012). This finding may lead to the development of new anti-EV71 drugs.

6.8.3. The 2B protein

Little is known about the role of the 2B protein in EV71 replication. However, the expression of 2B from polioviruses and coxsackieviruses can increase the permeability of the cell membrane to the translational inhibitor hygromycin B (Van Kuppeveld et al., 1997; Doedens and Kirkegaard, 1995). A study that focused on the 2B protein of coxsackievirus B3 showed that it can facilitate virus release, by increasing the concentration of free cytosolic Ca^{2+} (Van Kuppeveld et al., 1996). Recent studies demonstrated that the picornavirus 2B, and its precursor 2BC protein, contain two hydrophobic regions, which are important in multimerization and integration into the membrane of the host Golgi and ER complex, producing virus-induced vesicles and forming the viroporin complex (DeJong et al., 2002, 2003, 2004, 2006, 2008).

6.8.4. Drugs targeting 2B

In a recent study, Xie et al. (2011) reported that the 2B protein from EV71 might mediate a chloride-dependent current in oocytes. 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), a chloride-dependent current inhibitor, potently inhibits virus production and virus-induced cytopathic effect in RD cells, which may provide a new approach for identifying potential anti-EV71 drugs.

6.8.5. The 2C protein

The 2C protein is one of the most conserved picornavirus non-structural proteins. It is believed to have NAPase activity (Racaniello et al., 2007). It appears to be associated with viral RNA in infected cells, through involvement with the formation or maintenance of the replication complex (Hogle et al., 1985). Tang et al. (2007) reported that the EV71 2C protein interacted with host protein reticulon 3, which is critical for viral replication.

The 2C protein has also been shown to interfere with TNF-α-mediated activation of the NF-*k*B signaling pathway, which plays a key role in the inflammatory response. Recently, Zheng et al. (2011) found that the expression of the EV71 2C protein significantly reduced TNF-α-mediated NF-*k*B activation through the inhibition of I*k*B kinase-β activation. A more detailed understanding of the role of the EV71 2C protein in counteracting the host immune response may provide novel ideas for developing antiviral strategies.

6.8.6. Drugs targeting 2C

Two adenosine analogs, metrifudil (*N*-(2-methylphenyl) methyl adenosine) and N^6-benzyladenosine, which showed IC_{50}s of 1.3 μM and 0.10 μM, respectively, against EV71, were thought to block virus replication via interaction with 2C (Arita et al., 2008) (Fig. 3). Drug-resistant mutants were isolated, and sequence analysis identified mutations at nucleosides 5050 and 5815 in 2C resistant to metrifudil and at nucleosides 4428 and 5048 in 2C resistant to N^6-benzyladenosine. However, the mechanism of inhibition by these compounds remains to be elucidated.

6.9. Activities of P3 region-encoded viral proteins

6.9.1. Protein 3A

The EV71 3A protein plays an important role in inhibiting cellular protein secretion and mediating the presentation of membrane proteins, through its effect on protein trafficking by the ADP-ribosylation factor (Arf) family, an important component of the membrane secretion pathway (Belov et al., 2005). Arf activity independently mediates the intracellular trafficking that occurs in membranous vesicles (Behnia and Munro, 2005). Protein 3A induces the recruitment of Arf proteins to membranes; a mutant version was unable to induce Arf translocation. (Belov et al., 2005).

6.9.2. Drugs targeting the 3A protein

Enviroxime is a benzimidazole derivative that inhibits the replication of rhinoviruses and poliovirus by targeting protein 3A (Heinz and Vance, 1996; DePalma et al., 2008a,b). Enviroxime was reported to have strong antiviral effects against EV71, with an EC_{50} of 0.15 μM. A functionally enviroxime-like compound, AN-12-H5, was found to inhibit EV71 infections with an EC_{50} of 0.55 μM (Arita et al., 2010). Mutant resistance assays have suggested that AN-12-H5 is a bifunctional inhibitor, which blocks replication by targeting 3A, and also inhibits an early stage of infection by targeting VP1 and VP3.

The compound TTP-8307 was identified as a potent inhibitor of the replication of several enteroviruses and rhinoviruses; all drug-resistant variants of CVA16 and EV71 had at least one amino acid mutation in the 3A protein. TTP-8307 effectively inhibited CVA16 (IC_{50} 0.085 μM,) but it showed weak activity against EV71

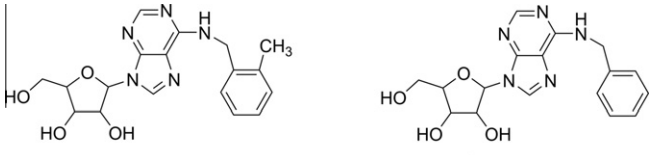


Fig. 3. Structure and biological activities of 2C protein inhibitors (Arita et al., 2008).

(EC_{50} > 60 μM). Despite the fact that 3A was identified as a prime target for both TTP-8307 and enviroxime, the precise mechanism of action for viral inhibition by these compounds remains to be elucidated (DePalma et al., 2009).

The compound GW5074, 3-(3,5-dibromo-4-hydroxybenzylidine-5-iodo-1,3-dihydro-indol-2-one), identified during the same screening as NF449, is a Raf-1 inhibitor that showed activity against EV71, with an IC_{50} of 2.0 μM and a CC_{50} of 170 μM (Arita et al.,2008). The target of GW5074 appeared to be different from those of *in vivo* neuroprotective action (B-Raf and ATF-3), but is apparently conserved among the enteroviruses; a later study found it to be the same region targeted by enviroxime in the protein 3A (Arita et al., 2009) (Fig. 4).

6.9.3. Protein 3B

EV71 utilizes the small virus-encoded 3B protein (also known as VPg) as a primer to initiate RNA synthesis by the $3D^{pol}$ (Paul et al., 1998; Semler, 2002). 3B is covalently linked to the 5′-terminus of all newly synthesized viral RNAs, via a phosphodiester bond between the terminal UMP in the viral genome and a conserved tyrosine residue at position 3 of protein 3B.

6.9.4. Protein 3C ($3C^{pro}$)

The EV71 $3C^{pro}$, a cysteine protease, plays a critical role in the generation of mature virion particles (Marcotte et al., 2007). Except for the cleavage of VP1/2A and 3C/3D by $2A^{pro}$, the $3C^{pro}$ protein is absolutely required for all EV71 polyprotein processing. $3C^{pro}$ reportedly enters nuclei through its precursor 3CD′ or 3CD, which contains a nuclear localization sequence (NLS) (Amineva et al., 2004; Sharma et al., 2004). Weng et al. (2009) found that the EV71 $3C^{pro}$ interferes with the polyadenylation of host cell RNA by digesting CstF-64, a critical host factor for 3′ pre-mRNA processing, suggesting a novel mechanism by which picornaviruses utilize $3C^{pro}$ to impair host cell function.

The picornavirus $3C^{pro}$ Can also cleave numerous factors and regulators associated with cellular DNA-dependant RNA polymerase I, II and III, such as the TATA-box-binding protein (TBP), octamer-binding protein (OCT-1), transcription activator p53, cyclic AMP-responsive element-binding protein (CREB), histone H3 and DNA polymerase III (Clark et al., 1991, 1993; Yalamanchili et al., 1997a,b,c; Falk et al., 1990; Weidman et al., 2001). $3C^{pro}$ may also be involved in virus-induced blockage of host transcription (Yalamanchili et al., 1997a,b,c). The cleavages of poly-A-binding protein by $2A^{pro}$ and $3C^{pro}$ also contribute to the inhibition of cellular translation (Kuyumcu-Martinez et al., 2002, 2004).

The crystal structures of the $3C^{pro}$ of several picornaviruses, such as HRV-2 (Matthews et al., 1994), FMDV (Birtley et al., 2005), poliovirus (Mosimann et al., 1997) and HAV (Bergmann et al., 1999) have been determined. The first crystal structure of the EV71 $3C^{pro}$ (PDB code: 3OSY) was determined by Cui et al. (2011), followed by its structure complexed with the inhibitor rupintrivir (PDB code: 3SJO; Wang et al., 2011), which reveals the roles of catalytically important residues. The EV71 $3C^{pro}$ has a typical chymotrypsin-like fold that is common for picornaviruses (Fig. 5). Conserved His 40, Glu71 and Cys 147 residues complete the EV71 catalytic triad. One striking difference is that a conserved structural moiety, the β-ribbon above the substrate binding cleft that forms parts of S2–S4 specificity pockets in the $3C^{pro}$ of other picornaviruses, adopts an unusual open conformation in the EV71 enzyme. Gly123 and His133, two important residues located at the base of the β-ribbon, form a hinge that governs the intrinsic flexibility of the ribbon (Cui et al., 2011).

6.9.5. Drugs targeting $3C^{pro}$

The essential role of $3C^{pro}$ in EV71 replication makes it an attractive target for drug discovery. Synthetic compounds targeting $3C^{pro}$ were initially designed based on the peptide substrate cleavage specificity of the enzyme. Many are peptides 3–5 amino acids and an aldehyde group, which serves as the electrophilic anchoring group (DePalma et al., 2008a,b). Modifications based on the enzyme structure were made on the compounds to obtain higher inhibitory and antiviral activity (i.e., SAR studies in combination with rational design). Replacement of a scissile amide carbonyl with a Michael acceptor can cause the formation of irreversible covalent bonds between the $3C^{pro}$ and peptidic 3C inhibitors (Dragovich et al., 1998).

Rupintrivir (AG7088), which reached Phase II clinical trials, is the most successful peptidomimetic $3C^{pro}$ inhibitor (Dragovich et al., 1999; Zhang et al., 2010a,b) (Fig. 6). It was designed to irreversibly inhibit the human rhinovirus (HRV) $3C^{pro}$ with broad-spectrum anti-picornavirus activity (Shih et al., 2004a,b,c). Binford et al.(2005) found that rupintrivir possessed antiviral activity

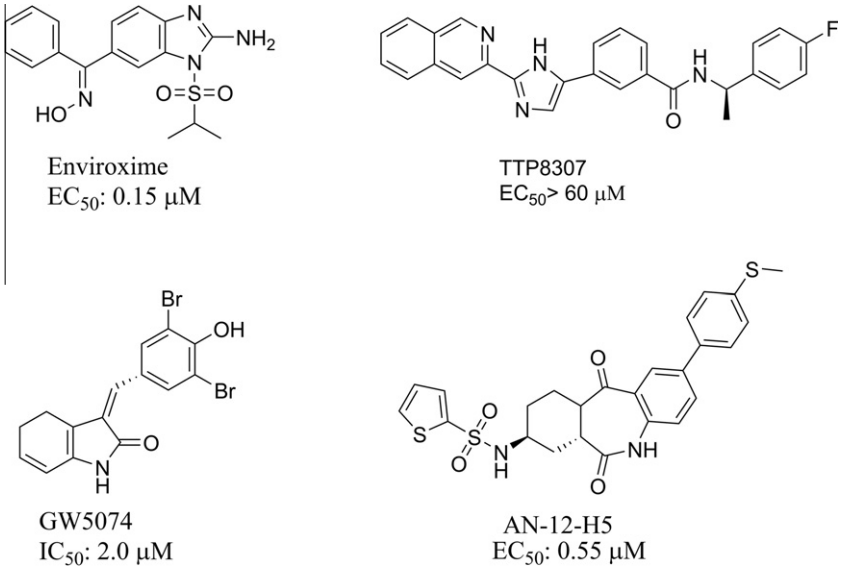


Fig. 4. Structures and biological activities of 3A protein inhibitors: Enviroxime (Heinz and Vance, 1996); TTP-8307 (DePalma et al., 2009); GW5074 (Arita et al., 2008); AN-12-H5 (Arita et al., 2010).

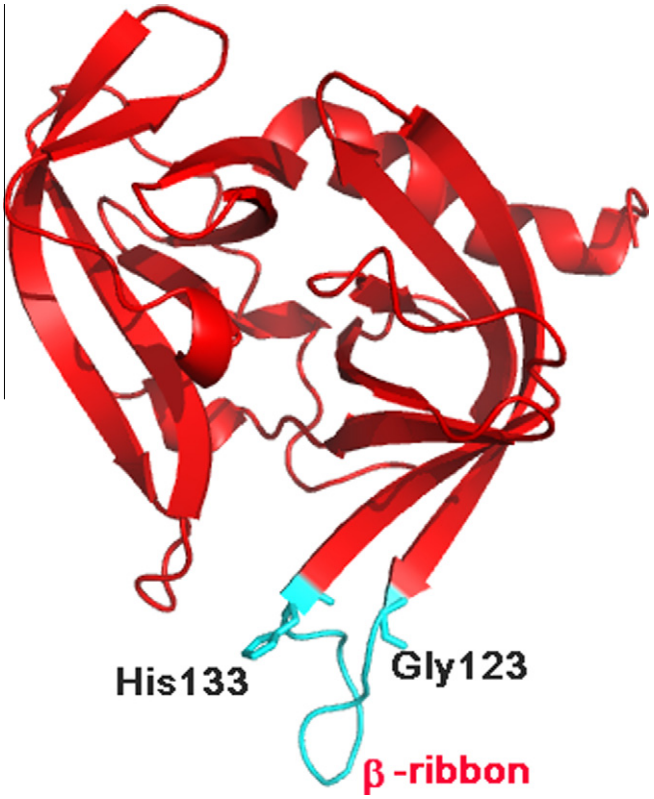


Fig. 5. Ribbon model of the structure of EV71 $3C^{pro}$ (drawn with PyMOL). The β-ribbon is indicated with an open conformation. The side chains of H133 and G123 are highlighted in stick presentation (Cui et al., 2011).

against 23 clinical isolates of HRV and 4 enterovirus strains (CVB2, CVB5, EV6 and EV9). Cui et al. (2011) showed that rupintrivir also inhibited EV71 $3C^{pro}$, with an IC_{50} of 2.3 μM. Rupintrivir also strongly synergized with IFN-α in inhibiting EV71 replication when tested in a replicon assay (Hung et al., 2011).

The crystal structure of the EV71 $3C^{pro}$ Complexed with rupintrivir reveals a half-closed S2 subsite and a size-reduced S1′ pocket, which limits the access of the α,β-unsaturated ester, the P1′ group of rupintrivir (Lu et al., 2011a,b; Wang et al., 2011). In order to accommodate the P1′ group of inhibitors, the α,β-unsaturated ethyl ester at the P1′ position was replaced by an aldehyde, and a series of potent rupintrivir analogues were developed by Kuo et al. (2008). The best inhibitor 10b (Fig. 6) exhibited a potent inhibitory activity, with an EC_{50} of 0.018 μM, without apparent toxicity (CC_{50} > 25 μM).

Several assays have also been developed to screen potential protease inhibitors (Mao et al., 2003; Lee et al., 2003; Lin et al., 2002; Tsai et al., 2009). Lee et al. (2008) reported a cell-based assay for high-throughput drug screening, which used a reverse two hybrid system to monitor the proteolytic activity of EV71 $3C^{pro}$.

6.9.6. Protein 3D ($3D^{pol}$)

The EV71 $3D^{pol}$ is a viral RdRp, one of the major components of the RNA replication complex. The $3D^{pol}$ of the Chinese epidemic strain was recently characterized biochemically (Jiang et al., 2011) and its ternary structure determined (PDB code: 3N6L) (Wu et al., 2010). Its overall crystal structure adopts a closed "right-hand" conformation, similar to other viral RdRps, which is composed of "fingers", "palm" and "thumb" domains (Fig. 7). Based on studies using the ribonucleotide analogue Br-UTP, the fingers domain is suggested to be involved in template binding, while the thumb should contribute to primer binding.

The $3D^{pol}$ uridylylates VPg and utilizes VPg-pUpU as a primer to initiate RNA replication (Paul et al., 1998, 2003). Polymerase oligomerization has been proposed to be responsible for efficient template utilization. Interestingly, the 3CD protein, the precursor of the mature $3C^{pro}$ and $3D^{pol}$, exhibits protease activity, but no polymerase activity (Harris et al., 1992).

6.9. 7. Drugs targeting $3D^{pol}$

Shih and coworkers (2004a,b,c) discovered piperazine-containing pyrazolo [3,4-d] pyrimidine derivatives as a class of antivirals against EV71. One such compound, DTriP-22, 4{4-[(2-bromo-phenyl)-(3-methyl-thiophen-2-yl)-methyl]-piperazin-1-yl}-1-pheny-1H-pyrazolo [3,4-d] pyrimidine) (Fig. 8), inhibited viral replication by reducing viral RNA accumulation, with an EC_{50} of 0.30 μM. The molecular target of this compound was identified by analyzing DTriP-22-resistant viruses. DTriP-22 suppressed the accumulation of both positive- and negative-stranded viral RNA during virus infection. Substitution of lysine for Arg163 in the EV71 $3D^{pol}$ rendered the virus drug resistant (Chen et al., 2009).

A group of polyanionic compounds, including aurintricarboxylic acid (ATA) (Fig. 8), inhibit several viruses in cell culture (Urbinati et al., 2008). ATA was reported to inhibit the adsorption, and thus

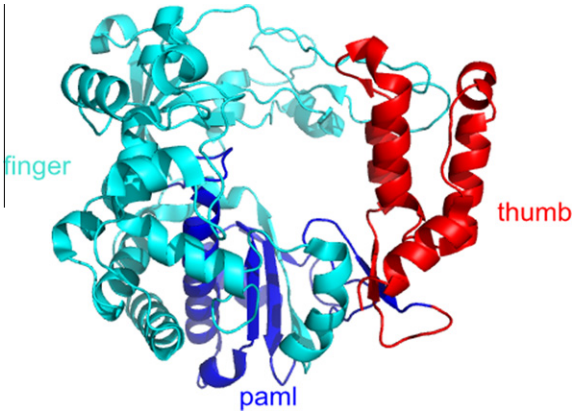
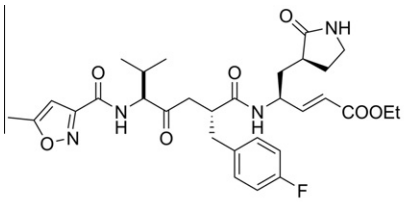
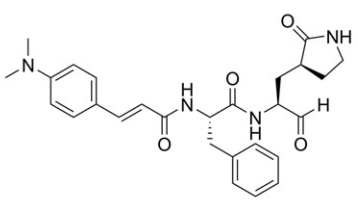


Fig. 7. Ribbon model of the structure of EV71 $3D^{pol}$ (drawn with PyMOL) is colored according to the referred crystal structure of $3D^{pol}$ (PDB code: 3N6L): finger in cyan, palm in blue, thumb in red (Wu et al., 2010).



Rupintrivir
IC_{50}: 2.3 μM



10b
EC_{50}:0.018 μM

Fig. 6. Structures and biological activities of 3C protease inhibitors: Rupintrivir (Dragovich et al., 1999); 10b (Kuo et al., 2008).

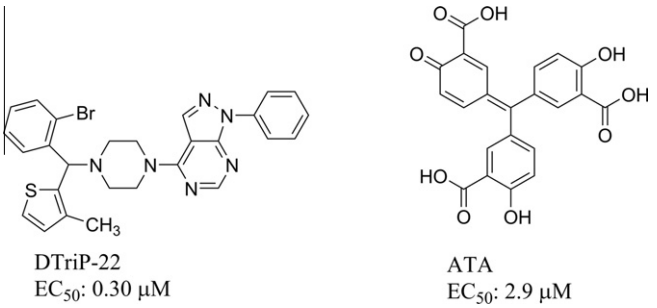


Fig. 8. Structures and biological activities of 3D protein inhibitors: DTriP-22 (Chen et al., 2009); ATA (Urbinati et al., 2008; Hung et al., 2010).

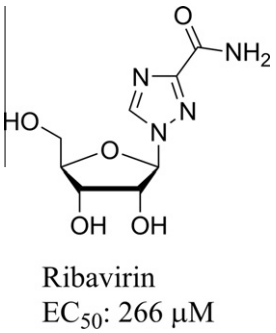


Fig. 9. Structure and biological activity of Ribavirin (Li et al., 2008).

block the replication of HIV by interfering with the interaction between the viral envelope glycoprotein (gp120) and the CD4 receptor on the cell surface (DeClercq et al., 2002). Recently, ATA was found to be a potent inhibitor of EV71 replication in an antiviral neutralization assay, and plaque assays demonstrated an EC_{50} of 2.9 μM. Studies of the mechanism of action revealed that ATA interferes with the viral $3D^{pol}$ (Hung et al., 2010).

7. Other inhibitors of EV71

7.1. RNA interference

RNA interference (RNAi), a native and specific post-transcriptional gene silencing mechanism, has been shown to inhibit the replication of poliovirus (Gitlin et al., 2002) and rhinovirus (Phipps et al., 2004), and is also effective against EV71. Various regions of the EV71 genome were targeted for inhibition by chemically synthesized siRNAs. Transfection of rhabdomyosarcoma (RD) cells with siRNA targeting the 3′UTR or the 2C, 3C, or 3D region significantly reduced viral cytopathic effect. The inhibitory effect was dose-dependent, with decreases in viral RNA, viral proteins, and plaque formation. No significant off-target effects were observed.

Selective inhibition of EV71 has also been demonstrated using plasmid-based shRNA (Lu et al., 2004; Wu et al., 2009). Recently, Deng et al. (2012) identified the highly conserved 5′UTR of EV71 genome as a target of siRNA to effectively inhibit viral replication. Transfection of 2′-modified siRNAs targeting the 5′UTR significantly delayed and alleviated cytopathic effects and increased cell viability in EV71-infected RD cells. Replication was also inhibited in the presence of specific siRNAs through knockdown of MEK1 expression (MAPK/ERK 1; MAPK: mitogen-activated protein kinase; ERK: extracellular regulated kinase) (Wang et al., 2012a,b).

7. 2. Type I interferon

Type I interferons (IFNs) exerted a direct protective effect on EV71-infected human cell lines (Liu et al., 2005). Of all IFN I subtypes, four IFNs (IFN-α4, IFN-α6, IFN-α14 and IFN-α16) displayed potent antiviral activity and IFN-α14 exhibited approximately 20 higher activity compared with conventional IFN-α2a (Yi et al., 2011). However, EV71 disrupted IFN signaling by reducing IFN receptor 1, which limits the IFN clinical application for the treatment of EV71 infected patients. The 2A protease encoded by EV71 has also been shown to play a crucial role in antagonizing the IFN response and its mechanism remains to be elucidated (Liu et al., 2012).

7.3. Ribavirin

Ribavirin, which has been used for the treatment of several different RNA virus infections, reduced the yields of EV71 *in vitro*, with an EC_{50} of 266 μM (Fig. 9). *In vivo* results showed that ribavirin reduced the mortality, morbidity and subsequent paralysis sequelae in EV71-infected mice by decreasing the viral load in tissues (Li et al., 2008). The compound is thought to enhance the mutation frequency of RNA viruses, resulting in "error catastrophe" and the loss of viability.

7.4. Chloroquine

Treatment of EV71-infected human glioblastoma (SF268) cells with chloroquine (Fig. 10), guanidine hydrochloride or cycloheximide prevented apoptosis, as characterized by DNA fragmentation and caspase activation (Shih et al., 2008). Moreover, treatment with 1.2 μM of chloroquine resulted in a 104-fold reduction of EV71 RNA synthesis. Investigations into the inhibitory mechanisms of chloroquine showed that a variety of targets and processes were involved (see Fig. 10)

7.5. Heparan sulfate

Heparan sulfate and its mimetics (heparin, pentosan polysulfate) exhibited significant antiviral actions at concentrations less than 250 mg/ml in *in vitro* anti-EV71 assay. The mode action assay

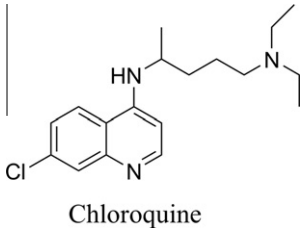


Fig. 10. Structure of chloroquine (Shih et al., 2008).

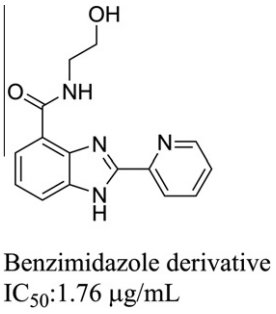


Fig. 11. Structure of benzimidazole (Xue et al., 2011). In the Fig. 11, IC_{50} is 1.76 micro g/mL, not 1.76 micro M. We have corrected it in the novel image of Fig. 11.

has shown that these compounds exerted antiviral activity through hindrance of viral attachment to the cells (Pourianfar et al., 2012).

7.6. Benzimidazole derivatives

Benzimidazole derivatives were screened in Vero cells against for several enteroviruses, including EV71 (Fig. 11). The most potent compound showed satisfactory antiviral activity (IC_{50} 1.76 μg/mL) with a remarkable selectivity index (328) (Xue et al., 2011) .

8. Natural products with anti-EV71 activity

Several natural compounds exhibit inhibitory activity against EV71 (Fig. 12). Allophycocyanin, a red fluorescent protein purified from the marine algae Spirulina platensis, has been found to prevent EV71-induced cellular apoptosis, delay viral RNA synthesis and reduce plaque formation, with an EC_{50} of 0.1 μM (Shih et al., 2003). Raoulic acid, purified from a whole-plant extract of a New Zealand plant, *Raoulia australis*, was tested for antiviral activity in Vero cells and inhibited EV71 with an EC_{50} of less than 0.1 μg/ml and a CC_{50} of more than 65 μg/ml, giving it a therapeutic index > 650 (Choi et al., 2009).

Ursolic acid is a triterpenoid purified from the aqueous extract of *Ocimum basilicum*, a herb commonly used in traditional Chinese medicine. Studies revealed post-infection inhibition of EV71 by lower doses of ursolic acid, but the exact mechanisms remain unclear (Chiang et al., 2005).

Treatment with lycorine reduced EV71 cytopathic effect in RD cells by inhibiting viral replication. Analysis suggests that lycorine blocks elongation of the viral polyprotein during translation. Lycorine treatment of mice challenged with a lethal dose of EV71 resulted in reductions in mortality, improved clinical scores and fewer pathological changes in the muscles, associated with inhibition of viral replication. When mice were infected with a moderate dose of EV71, lycorine treatment prevented paralysis (Liu et al., 2011).

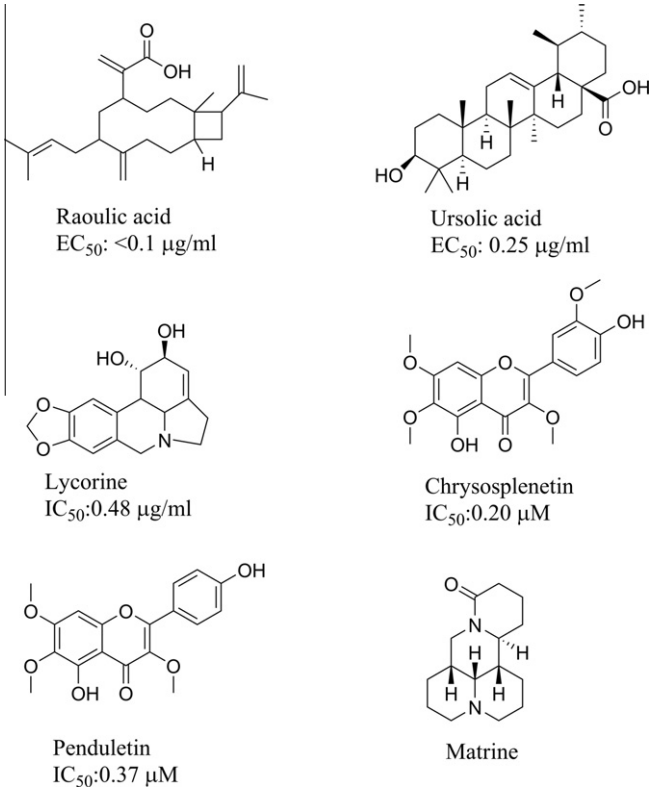


Fig. 12. Structure and biological activities of natural products: Raoulic acid (Choi et al., 2009); Ursolic acid (Chiang et al., 2005); Lycorine (Liu et al., 2011); Chrysosplenetin, Penduletin (Zhu et al., 2011); Matrine (Yang et al., 2012).

Chrysosplenetin and pendulentin, two flavonols isolated from the leaves of *L. pterodonta*, showed strong activity against EV71 in Vero and RD cell-based infection systems. Preliminary assays found that both compounds inhibited viral RNA replication (Zhu et al., 2011). Another natural product, matrine, is an alkaloid with inhibitory activity against EV71. It reduced the mortality and reduced signs of illness in mice challenged with a lethal dose of EV71 (Yang et al. 2012).

9. Conclusions

The increased frequency of HFMD outbreaks is one of the most pressing health concerns in China and many Asian countries, indicating the need for effective antiviral therapies. A number of compounds with a variety of mechanisms of action have been shown to inhibit EV71 replication, but none has been advanced to human clinical trials. Recent success in developing drugs against hepatitis C virus should offer valuable experience for anti-EV71 drug development. The $3C^{pro}$ inhibitor rupintrivir, which has been extensively characterized in various stages of clinical trials, may provide a good starting point for the development of new drugs. The crystal structures of $3C^{pro}$ and $3D^{pol}$ can also be used for the rational design of inhibitors (Cui et al., 2011; Wu et al., 2010). There thus appear to be many opportunities for the development of effective antivirals against EV71.

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