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REVIEW ARTICLE

Development of antiviral agents toward enterovirus 71 infection

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Enterovirus 71 (EV71) infection remains a public health problem at a global level, particularly in the Asia-Pacific region. The infection normally manifests as hand–foot–mouth disease; however, it is capable of developing into potentially fatal neurological complications. There is currently no approved vaccine or antiviral substance available for the prevention or treatment of EV71 infection. This paper, thus, reviews efforts to develop or discover synthetic as well as naturally occurring compounds directed against EV71 infection. The recent achievements in cellular receptors of EV71 are also highlighted, and their contribution to the development of antiviral drugs against EV71 is discussed in this article.
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Introduction

Enterovirus 71 (EV71) is a pathogenic serotype of the Picornaviridae family, containing a single positive sense RNA genome with a length of approximately 7.5 kb. Detected during a small outbreak in California between 1969 and 1972,[1] EV71 has now been turned into one of the most pathogenic Enterovirus serotypes with many outbreaks occurring around the world, particularly in the Asia-Pacific region. Although EV71 infection usually manifests with rashes and vesicular lesions on the hands, feet, and oral mucosa,[2] it sometimes leads to fatal neurological complications such as aseptic meningitis, encephalitis, acute respiratory disease, and pulmonary edema.[3] Following the large outbreaks in both Malaysia and Taiwan in the late 1990s,[4] new life-threatening outbreaks of EV71 reoccurred in Taiwan in 2001–2002[5] and in China in 2010.[6]

There is no approved vaccine or antiviral drug available for prophylaxis or treatment of EV71 infection to date.[7] Therefore, research studies should continue to develop

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novel EV71 inhibitors and as such, latest achievements in this field always have to be updated. At least three recent reviews have detailed a range of studies undertaken in both fields of EV71: antivirals and vaccines.[7–9] However, compared to EV71 vaccine development, a large number of research ventures conducted into EV71 antiviral development has generated vast information on novel compounds with anti-EV71 capacity and even improved the understanding of EV71–host interactions. Hence, this review attempts to provide a focused body of information regarding the status and challenges of antiviral therapy for EV71, covering both options: synthetic and natural bioactive compounds. In addition, we discuss how newly discovered EV71 cellular receptors might lead to new avenues for anti-EV71 drug design.

EV71 structural protein inhibitors

It is well known that variations within capsid proteins VP1–VP3 of EV71 are responsible for the antigenic diversity of the virus, whereas neutralization sites are most densely clustered on VP1.[10] Thus, EV71 VP1 inhibitors have been suggested as one of the first candidates for developing antivirals against the viral infection. Thus far, pleconaril has been known as a viral capsid binder that has exhibited inhibitory effects against a number of Enteroviruses[11,12] except EV71.[10] However, a recent study interestingly showed that pleconaril could significantly increase the viability of EV71-infected cells and reduce the mortality of EV71-infected mice.[13] Therefore, it remains to be investigated whether pleconaril should be considered a potent EV71 inhibitor.

Among other capsid binders, pyridyl imidazolidinone was the first to demonstrate notable potencies against EV71 infection in a number of consecutive studies.[10,14–17] Pyridyl imidazolidinone is a new category of capsid binders, generated by a computer-assisted drug design.[10] It is believed that pyridyl imidazolidinone exerts its antiviral action by fitting into the viral hydrophobic pocket of VP1.[17] In the pyridyl imidazolidinone family, BPROZ-101 with a half-maximal inhibitory concentration (IC_{50}) of 0.0012 ± 0.0005 (μM) and BPROZ-074 with an IC_{50} of 0.0008 ± 0.0001 (μM) exhibited the most considerable antiviral activities against EV71.[17] In addition to pyridyl imidazolidinone, an EV71 VP1-derived peptide was also demonstrated to reduce the cytopathic effects of all EV71 strains from genotypes A, B, and C, with IC_{50} values ranging from 6 mM to 9.3 mM in RD cells.[18]

EV71 nonstructural protein inhibitors

The seven nonstructural proteins of EV71 are the essential elements involved in various viral functions including proteases, RNA replication, ATPase activity, RNA helicase activity, and RNA replication. Hence, these peptides have been studied as potential targets for designing antiviral agents toward EV71.[19]

$2A^{pro}$

Recently, it was shown that a six-amino-acid peptide, LVLQTM, exhibited antiviral potencies against EV71 in HeLa cells. This peptide was shown to serve as a substrate mimetic of the EV71 $2A^{pro}$ that is known to be responsible for the viral protease activity.[20]

2B

Little information is available for the function of the 2B peptide of EV71, but it is thought to be important for EV71 RNA replication.[21] In this regard, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid was shown to prevent EV71 2B activity, leading to the inhibition of virus production in RD cells.[22]

2C

As a multifunctional protein, EV71 2C has been suggested to be involved in processing nucleoside triphosphatase activity[23] and synthesis of RNA negative strands.[24] Two adenosine analogues—Metrifudil and N^6-benzyladenosine—have been demonstrated to interact with the EV71 2C peptide and inhibit viral infection.[25]

3A

EV71 3A is highly conserved and serves as a scaffold of the viral RNA replication complex.[19] In this respect, an enviroxime mimetic compound, AN-12-H5, was reported to target the 3A region of EV71 and significantly inhibit an early stage of EV71 infection after virus binding.[26]

$3C^{pro}$

The EV71 3C peptide acts as a viral protease during the viral infection of host cells, playing a vital role in the maturation of virion particles. A study showed that rupintrivir promisingly inhibited EV71 3C by mimicking the substrate of the 3C protease.[27] The anti-EV71 activity of rupintrivir was also evaluated *in vivo*,[28] where the rupintrivir-treated suckling mice were largely protected from EV71-caused limb paralysis. Because of its safety, it has been concluded that rupintrivir can be suitable for immediate evaluation as a therapy in EV71-infected individuals with fatal neurological complications.[28]

$3D^{pol}$

EV71 $3D^{pol}$ is an RNA-dependent RNA polymerase that serves as a key element for the viral RNA replication process. DTriP-22 as a nonnucleoside analogue was shown to inhibit EV71 infection by reducing the viral RNA accumulation after virus absorption.[29] In addition to DTrip-22, aurintricarboxylic acid (a polyanionic compound) could prevent EV71 infection through interference with $3D^{pol}$ in Vero cells.[30]

Nucleotide analogues

Ribavirin (RBV) in the form of ribavirin triphosphate can serve as a base analogue of either ATP or GTP. The antiviral potency of RBV has been presented for a wide range of RNA viruses *in vitro* and/or *in vivo*,[31–39] including EV71[40] where

it reduced the EV71 titer in human and mouse cell lines. It also decreased the rate of death, morbidity, and succeeding paralysis sequelae in EV71-infected mice.[40]

In contrast with the afore-mentioned findings, a recent study showed that RBV failed to protect 1-day-old mice infected with EV71.[13] Additionally, some EV71 strains were even resistant to RBV at concentrations much higher than those reported previously.[18] In our studies, RBV (applied as a positive control) could not significantly prevent the EV71 infection in Vero cells and even showed a high degree of cytotoxicity.[41] Thus, further studies are warranted to test the usefulness of RBV as a positive control in different cell types and for different EV71 strains.

RNA interference

RNA interference has effectively been exploited as an antiviral tool against EV71 infection *in vitro*.[42–45] In addition to *in vitro* studies, promising results have been attained through an *in vivo* study.[46] The findings revealed that the mice treated with 10 nM of 19-mer short interfering RNAs or 50 μg of the plasmid-derived 19-mer small hair pin RNAs did not develop signs of weight loss or hind limb paralysis up to 14 days post infection.[46]

Heparan sulfate mimetics

Heparan sulfate (HS) is present in extracellular matrix, cell surfaces, and intracellular granule secretions of all types of animal tissues.[47] HS is composed of a basic structure of sulfated polymers of alternating uronic acid-(1→4)-D-glucosamine.[48] HS mimetics are a group of soluble synthetic or semisynthetic compounds, structurally related to cellular HS, which are able to simulate the functions of cell surface HS.[49] Because cellular HS serves as a carbohydrate receptor for the human viruses of various families,[50] HS mimetics are thus assumed to simulate the function of a cellular receptor for many of viruses.[51] Therefore, HS mimetics have significant applications in virology, particularly for development of antivirals and determination of viral receptors.[52]

The antiviral potencies of HS mimetics against EV71 were recently demonstrated by our group.[41] Several soluble HS mimetic compounds, including heparin, pentosan polysulfate, and HS, were shown to exhibit potent antiviral activities against a cloned strain of EV71 in Vero cells at noncytotoxic concentrations less than 250 μg/mL, as opposed to RBV as the positive control. The mode of action assays revealed that all the compounds were capable of exerting antiviral activities by hindering viral attachment to the cells.[41]

In order to gain insight into the mechanism(s) of action of heparin against EV71 infection, we followed our studies with a genome-wide microarray analysis of an EV71-infected human neural cell line, SK-N-SH. To make the results more relevant and applicable, microarray analysis was carried out concurrently with antiviral activity and cytotoxicity tests of heparin, analyzed through a multilevel comparison. Of the more than 30,000 genes studied, 14 well-known annotated genes were selected for which heparin significantly modified the intensities of genes that have already been upregulated or downregulated by EV71.[53]

Type I interferon subtypes

Following the demonstration of a protective effect for type I interferons (IFNs) toward EV71 infection,[54] anti-EV71 potencies of 17 type I IFNs were assayed in Vero cells, where IFNs α4, α6, α14, and α16 exhibited more antiviral activities compared with the rest.[55] However, further investigations by the same research group revealed that EV71 was able to suppress the cellular type I IFN response by reducing IFN receptor 1 level. Moreover, EV71 $2A^{pro}$ was shown to play a crucial role in antagonizing the type I IFN response. These new findings shed light on how EV71 might suppress the host innate immune response, and thus may assist the development of new therapy options for EV71 infection.[56]

Antiviral peptides

Thus far, at least one natural cationic peptide—lactoferrin—has been reported to exert antiviral activities toward EV71 infection.[57] Lactoferrin is an iron-binding glycoprotein present in milk, saliva, mucous secretions, and other biological fluids of mammals. Both bovine and human lactoferrins significantly prevented infection of isolates of EV71 in RD cells with an IC_{50} of 10.5–24.5 μg/mL and 103.3–185.0 μg/mL, respectively. Further *in vivo* experiments demonstrated that lactoferrin saved 17-day-old ICR mice from fatal EV71 challenge.[58] It was proposed that lactoferrin exerts its antiviral action through the prevention of viral entry by blocking cellular receptors and/or by direct binding to the virus.[58]

Other synthetic EV71 inhibitors

N-phenylbenzamide

A series of *N*-phenylbenzamide derivatives were synthesized, and their antiviral potencies were evaluated against four strains of EV71—SZ-98, JS-52-3, H, and BrCr—in Vero cells, where 3-amino-*N*-(4-bromophenyl)-4-methoxybenzamide effectively showed an IC_{50} value ranging from 5.7 ± 0.8 μM to 12 ± 1.2 μM.[59]

7-Hydroxyisoflavone

It was shown that 7-hydroxyisoflavone dose-dependently exhibited a potent antiviral activity against three different EV71 strains at an early step of EV71 replication.[60]

Methyl 3,4-dihydroxyphenylacetate

EV71 replication in RD cells was shown to be prevented by methyl 3,4-dihydroxyphenylacetate. The compound, at a concentration of 0.01 μg/μL, reduced EV71 VP1 mRNA 48 hours post infection, with a low toxicity on RD cells.[61]

BPR3P0128

BPR3P0128, a novel compound with potent inhibitory effects against influenza, was also shown to significantly inhibit infection of some RNA viruses, including EV71.[62]

FLICE-like inhibitory protein

FLICE-like inhibitory protein (FLIP) has been known as a negative regulator of death ligand-directed apoptosis, which modulates virus pathogenesis. It was reported that EV71 replication was noticeably reduced in MRC5 cells preincubated with anti-FLIP peptides.[63]

Benzimidazole derivatives

A study showed that one of the screened benzimidazole derivatives inhibited EV71 infection in Vero cells, with a low cytotoxicity and an IC_{50} of 1.76 μg/mL.[64]

N-arylethyl isoquinoline derivatives

The antienteroviral activities of a series of synthesized *N*-arylethyl isoquinoline derivatives were tested *in vitro*. Among these agents, the compound 7f exerted a significant antiviral activity against Coxsackievirus B3, whereas it exhibited a moderate activity toward EV71.[65]

Chloroquine, guanidine hydrochloride, and cycloheximide

Three compounds—chloroquine, guanidine hydrochloride, and cycloheximide—were shown to prevent EV71-mediated apoptosis in human glioblastoma cells. Interestingly, 1.2 l mM of chloroquine could reduce EV71 RNA synthesis more than 90 times.[66]

Anti-EV71 capacity of phytochemicals

Medicinal plants are considered rich and as yet fully unexploited sources of novel phytochemicals, which are worth being considered as potential antiviral drugs. Thus far, a number of naturally occurring compounds have been reported to have antiviral activities toward EV71 infection. Table 1 lists a number of studies undertaken to examine the antiviral capacities of several plant- or algae-derived compounds against EV71 infection.[67–84] Many of these reputable medicinal herbs have a long history of use in traditional Chinese medicine, which makes them more applicable for further investigations and less problematic for humans. Thus, it is not surprising that such research studies have mostly been undertaken by scientists from the Asia-Pacific region, where several fatal outbreaks of EV71 have occurred.

Apart from the differences in their antiviral potencies, phytochemicals tested for EV71 antiviral activity featured several common characteristics; for example, almost all of them were shown to exert low cytotoxicity in their antiviral doses. Whereas the stage in which EV71 is inhibited has been determined for all compounds, an exact molecular mechanism(s) of action has been drawn for only some of them, including aloe-emodin,[71] the extract of *Houttuynia cordata*,[72] kappa carrageenan,[75] the leaf extract of *Kalanchoe gracilis*, and the extract of *Paris polyphylla* Smith.[79] Another aspect is that recent studies on anti-EV71's influence of plant-derived compounds have also been performed in an animal model[77,80–84] (Table 1).

Screening among local medicinal plants could also be considered an effective and fast option for the discovery of anti-EV71 bioactive compounds. In this respect, a study reported screening 22 Chinese herbs for their anti-EV71 potentialities, leading to selection of the plant, *H. cordata*.[72] Consistent with these findings, another study attempted to screen among 12 commonly used antiviral herbs that were recommended by Chinese government agencies for the hand–foot–mouth disease. The results interestingly revealed that *H. cordata* was the only remedy with a potent antiviral activity against both EV71 and Coxsackievirus A16.[73] These findings might warrant follow-up studies to investigate the exact molecular mechanisms of action and evaluate the anti-EV71 capacity of this plant in an animal model.

EV71 receptors and their contribution to antiviral development

To date, several molecules have been identified and characterized to serve as cellular receptors for human enteroviruses. The most common enteroviral receptors include glycoproteins such as decay-accelerating factor and the Coxsackievirus and adenovirus receptor, which mediate infection for many enteroviral serotypes particularly echoviruses[85] and group B Coxsackieviruses.[86] Whereas there is concrete evidence of viral receptors of human enteroviruses, the discovery of the possible cell receptors of EV71 does not have a long history. In fact, the cellular receptors of EV71 were not clearly elucidated until 2009, when two research groups independently reported the discovery of two different receptors for the virus: the human scavenger receptor class B, member 2 (SCARB2)[87] and the human P-selectin glycoprotein ligand-1 (PSGL).[88]

Since the outstanding discoveries of EV71 receptors,[87,88] other molecules have also been suggested as candidate cellular receptors for EV71, including sialic acid[89,90] and DC-SIGN.[91] Furthermore, it was also proven that EV71 utilizes cell surface HS as an attachment receptor in RD cells.[18] Compliant with this finding, our research studies showed that the highly sulfated domains of cellular HS may serve as an essential attachment coreceptor for a clinical isolate of EV71 in Vero cells.[92] Later, our DNA microarray analysis proved that the heparan sulfate 2-*O*-sulfotransferase 1 (*HS2ST1*) gene was significantly upregulated by the EV71 infection of neural cells.[53] This gene has a role in the biosynthesis of cell surface HS. These findings supported the speculation that clinical EV71 required cell surface HS to infect or bind neural cells, in agreement with the recent discoveries of the role of HS in mediating EV71 infection.[18,92]

Although it remains to be investigated how the discovered receptors might contribute to the neurotropism of EV71, these findings could have implications for the design

Table 1 Anti-EV71 capability of several bioactive compounds from plants and algae

Source/reference	Active component	Use in folk medicine	*In vitro* mechanisms of action	*In vivo* assays
Salvia miltiorrhiza (Danshen)[67]	Crude extracts of dried roots	TCM	Interference with viral entry	—
Ocimum basilicum (sweet basil)[68]	Ursolic acid	TCM	Inhibition of viral replication after infection	—
Five medicinal plants[69]	Named Sheng-Ma-Ge-Gen-Tang[a]	TCM	Inhibition of viral attachment and penetration	—
Pueraria lobata Ohwi[70]	Named Ge-Gen-Tang	TCM	Inhibition of EV71 before and after infection	—
Rheum palmatum[71]	Aloe-emodin	TCM	A potent interferon-inducer	—
Houttuynia cordata[72,73]	Water extracts	TCM	Suppressing EV71-induced caspase 3 activation	—
Spirulina platensis (a blue-green alga)[74]	Allophycocyanin	—	More effective prior to viral infection	—
seaweeds[75]	Kappa carrageenan	—	Forming virus-polysaccharide complexes to prevent viral entry	—
Glycyrrhiza uralensis Fisch[76]	Glycyrrhizic acid	TCM	suppressing EV71 replication after virus entry	—
Kalanchoe gracilis[77]	Leaf extracts[b]	TCM	Inhibiting EV71 2A proteases, reducing EV71-activated caspase 9, decreasing EV71-induced up-regulation of IL-6 and RANTES genes	Inhibiting EV71 replication in the intestine of suckling mice
Daphne genkwa[78]	Extracts of dry flower buds	TCM	Targeting EV71 entry	—
Paris polyphylla Smith[79]	Ethanol extracts	TCM	Increasing amounts of IL-6 production	—
Several medicinal plants[80]	Punicalagin[c]	TCM	Reducing EV71-caused viral CPE	Reducing mortality of EV71-challenged mice
Several medicinal plants[81]	Matrine[c]	TCM	Suppressing EV71 RNA copy number	Reducing mortality of EV71-challenged mice
Several medicinal plants[82]	Geraniin[c]	TCM	Reducing EV71-caused viral CPE	Reducing mortality of EV71-challenged mice
Laggera pterodonta[83]	Chrysosplenetin and penduletin	TCM	Inhibition of EV71 after infection	—
Terminalia chebula[84]	Chebulagic acid	TCM	Reducing EV71-caused viral CPE	Reducing mortality of EV71-challenged mice

[a] Sheng-Ma-Ge-Gen-Tang is a mixture of crude extracts of five medicinal plants: including *Pueraria lobata* Ohwi, *Paeonia lactiflora* Pallas, *Cimicifuga foetida* L., *Glycyrrhiza uralensis* Fisch, and *Zingiber officinale* Roscoe.
[b] Some of the active components were determined to be ferulic acid, quercetin, and kaempferol.
[c] The pure compounds—punicalagin, matrine, and geraniin—can be found in medicinal plants such as *Punica granatum*, *Sophora* genus, and geraniums, respectively.
CPE = cytopathic effect; EV71 = enterovirus 71; TCM = traditional Chinese medicine.

or development of anti-EV71 agents. In this respect, one straightforward approach would be designing a monoclonal antibody or a soluble form of a receptor in order to recognize, mimic, and/or block the receptor. The examples include the monoclonal antibody and/or soluble forms of SCARB2,[87] PSGL-1,[88] and DC-SIGN antibody,[91] all of which have shown potent abilities to block EV71.

Sialic acid-mediated EV71 attachment might suggest that natural sialic acids in human milk could have a role in preventing EV71 infection in infants, in addition to other antibodies present in human milk.[89] Likewise, the essential role of cellular HS in EV71 binding would draw attention to HS mimetic compounds as candidate anti-EV71 agents.[41] In this respect, a variety of synthetic HS derivatives could be designed in order to evaluate or improve their anti-EV71 capabilities.

Review of the literature illustrates that none of the reported anti-EV71 compounds has been subjected to human

clinical trials. However, some synthetic compounds have promisingly been suggested as potential start points, such as the $3C^{pro}$ inhibitor, rupintrivir.[9] This compound has extensively been characterized in various stages of clinical trials for other viruses, and may thus be suitable for further evaluation of its potential benefits in EV71-infected patients.[9]

Nowadays, drug discovery from natural products may no longer be very attractive for global pharmaceutical companies because of business considerations and the perception that it is not cutting-edge research.[93] This issue might be also the case for plant-derived anti-EV71 compounds. However, in countries with rich sources of ethnopharmaceuticals, it is still reasonable to evaluate natural products as a complementary approach with promising perspectives. Such studies would be better directed toward phytochemicals with a convincing record of use in folk medicine. Moreover, drawbacks such as complexity of active components, the lack of information on mechanisms of action, and the lack of an *in vivo* assay should be overcome. Among the anti-EV71 natural compounds studied in an animal model, one promising candidate would be the active components of *K. gracilis*,[77] for which an inhibitory effect on EV71 2A proteases was determined.

Genome-wide analyses such as DNA microarrays could assist scientists to determine targets and pathways for a purported antiviral compound. However, such studies have apparently been limited with EV71; these research efforts include a study of the expression patterns of EV71 infection[94] and our recent microarray study in which the effect of heparin on the expression profiles of EV71-infected cells was analyzed.[53]

The recent discoveries of cell receptors of EV71 have shed light into the pathogenesis of the virus. Use of receptor antagonists is a well-known approach for designing drugs against a variety of disorders, including infectious viruses. Hence, newly discovered EV71 receptors could be considered promising, putative drug targets against viral infection.

Conflicts of interest

The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in the manuscript.

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