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Review Article

Recent Advances in Enterovirus A71 Infection and Antiviral Agents

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ABSTRACT

Enterovirus A71 (EV-A71) is one of the major causative agents of hand, foot, and mouth disease (HFMD) that majorly affects children. Most of the time, HFMD is a mild disease but can progress to severe complications, such as meningitis, brain stem encephalitis, acute flaccid paralysis, and even death. HFMD caused by EV-A71 has emerged as an acutely infectious disease of highly pathogenic potential in the Asia-Pacific region. In this review, we introduced the properties and life cycle of EV-A71, and the pathogenesis and the pathophysiology of EV-A71 infection, including tissue tropism and host range of virus infection, the diseases caused by the virus, as well as the genes and host cell immune mechanisms of major diseases caused by enterovirus 71 (EV-A71) infection, such as encephalitis and neurologic pulmonary edema. At the same time, clinicopathologic characteristics of EV-A71 infection were introduced. There is currently no specific medication for EV-A71 infection, highlighting the urgency and significance of developing suitable anti–EV-A71 agents. This overview also summarizes the targets of existing anti–EV-A71 agents, including virus entry, translation, polyprotein processing, replication, assembly and release; interferons; interleukins; the mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase B signaling pathways; the oxidative stress pathway; the ubiquitin-proteasome system; and so on. Furthermore, it overviews the effects of natural products, monoclonal antibodies, and RNA interference against EV-A71. It also discusses issues limiting the research of antiviral drugs. This review is a systematic and comprehensive summary of the mechanism and pathological characteristics of EV-A71 infection, the latest progress of existing anti–EV-A71 agents. It would provide better understanding and guidance for the research and application of EV-A71 infection and antiviral inhibitors.

Introduction

Human enteroviruses are members of the genus *Enterovirus* in the *Picornaviridae* family, which comprises a group of small, nonenveloped, positive-sense single-stranded RNA viruses. Enterovirus A71 (EV-A71) belongs to the human enterovirus species. EV-A71 can cause hand, foot, and mouth disease (HFMD) among children aged <5 years owing to their underdeveloped immune systems, according to the Chinese guidelines for diagnosing and treating HFMD.[1] Its clinical manifestation is milder, usually with fever, oral ulcers, and skin rashes on the hands and feet. However, HFMD caused by EV-A71 infection sometimes accompanies severe neurologic complications (Fig. 1), such as

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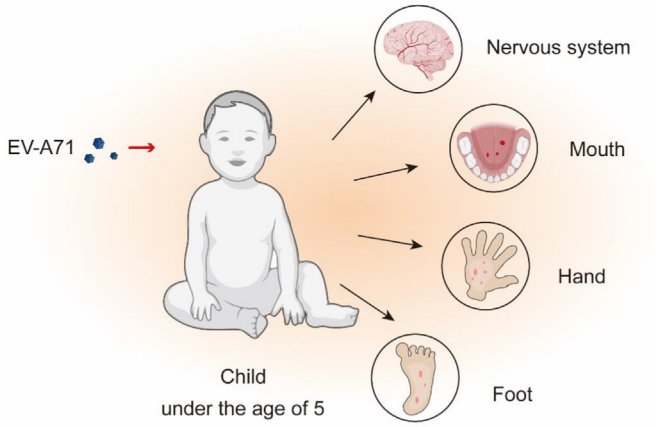

Figure 1.
Clinical manifestations of EV-A71 infection. The clinical features of EV-A71 are oral ulcers and skin rashes on the hands and feet. EV-A71 infection sometimes causes severe neurologic disorders, such as aseptic meningitis and encephalitis. EV-A71, enterovirus A71.

aseptic meningitis, encephalitis, poliomyelitis-like paralysis, and even death.

In 1969, enterovirus 71 (EV-A71) was detected in the United States in a 9-month baby with encephalitis. The virus was also identified in California,[2] and after that, it spread across the world, especially in the Asia-Pacific region,[3-7] including China,[6,7] Singapore,[8] Vietnam,[9] Japan,[10] and so on. EV-A71 caused 126 deaths in 2008 in China,[11] and the largest outbreak in China occurred in 2010, with an estimated 1.7 million infections, of which 27,000 caused severe neurologic complications, resulting in 905 fatalities.[12] Fatal cases were also reported in Asia as recently as 2012.[13]

EV-A71 produces a broad spectrum of clinical manifestations. The majority of infected individuals have asymptomatic infection. Mild cases are characterized as cutaneous diseases, such as HFMD and herpangina. Occasionally, EV-A71 infection can cause serious neurologic diseases, even life-threatening. Currently, EV-A71 vaccines are available on the Chinese market. However, owing to the mutating nature of RNA viruses, these vaccines may not provide long-term protection. Moreover, the vaccine mainly plays a preventive role, and the therapeutic drugs specific to EV-A71 in clinical use are still blank.[14] Therefore, there is an urgent need for anti−EV-A71 drugs to combat HFMD. This review summarized the pathological mechanisms caused by EV71 infection and focuses on the whole process of EV-A71 infection and related host factors, and classifies antiviral agents targeting EV-A71 into 6 major categories, namely inhibitors of entry, translation, polyprotein processing, replication, assembly, and release, and other inhibitors (Fig. 2).

The Properties and Life Cycle of the Virus

The EV-A71 virus is nonenveloped and icosahedral with a diameter of approximately 20 to 30 nm and a single-stranded positive polarity (+) RNA genome. The life cycle of EV-A71 (Fig. 3) starts with binding to one or more receptors on the cell surface. When EV-A71 binds to receptors, structural changes occur in the viral capsid and cell membrane. Then, partial pores are formed in the cell membrane to facilitate the penetration of the virion's positive-strand (+) RNA genome into the cytoplasm. The viral protein VPg (3B) acts as a primer for viral replication and

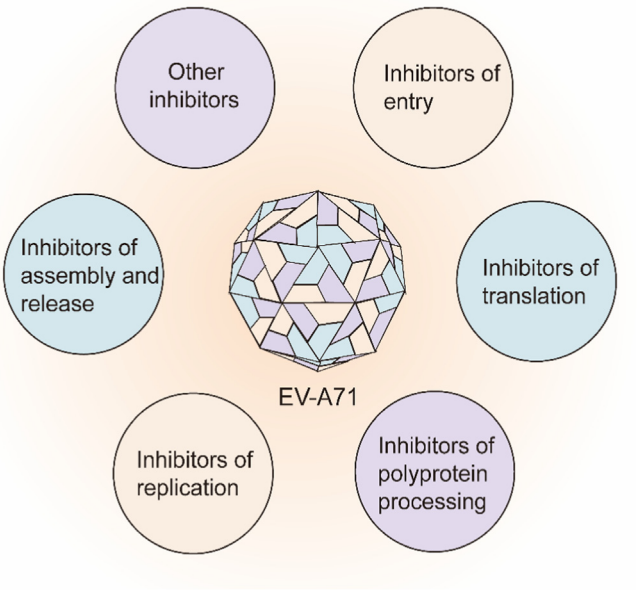

Figure 2.
Classification of antiviral agents for enterovirus A71 (EV-A71) infection. There are 6 major categories of anti-EV-A71 agents, namely inhibitors of entry, translation, polyprotein processing, replication, assembly and release, and other EV-A71 inhibitors.

covalently links to the EV-A71 genome. The parent virus RNA is a messenger RNA and it is translated into a single large polyprotein. Next, the viral protease 2A processes the polyprotein into the following 3 parts: 1 structural (P1) and 2 nonstructural (P2 and P3) regions. Among these 3 regions, P1 is further proteolyzed into VP0 (VP2 and VP4), VP1, and VP3. P2 and P3 are ultimately converted into 7 replicate proteins (P2-2A, -2B, and -2C; P3-3A, -3B, -3C, and -3D) by viral proteinases 3C and 3CD.[15]

The virus uses the error-prone RNA-dependent RNA polymerase 3D to replicate the viral genome in a vesicular membrane structure. The virus uses the negative-strand (−) RNA as a template to synthesize new positive-strand (+) RNA. The newly generated positive-strand (+) RNA enters the following steps of the viral life cycle: transcription, translation, and replication. Then, the newly synthesized genomic RNA binds to the viral capsid proteins to form new virions—virus maturation heralds when VP0 is genome inducible for cleavage into VP4 and VP2. Finally, mature viral particles are released extracellularly through cellular lytic or nonlytic pathways.[16]

Pathogenesis and Pathophysiology of Enterovirus A71 Infection

Virus Infection Path

The mechanism of EV-A71 pathogenesis has been studied extensively. Humans are the only host and source of infection for EV-A71. EV-A71 infects an organism and initiates the viral replication cycle after successfully binding to specific receptors on the host, such as gastrointestinal, respiratory, and dendritic cells. EV-A71 initially replicates in the lymphoid tissue of the oropharyngeal cavity and small intestine. Further, it proliferates in the deep cervical and mesenteric nodes before entering the reticuloendothelial system, heart, lungs, skin, mucous membranes, and central

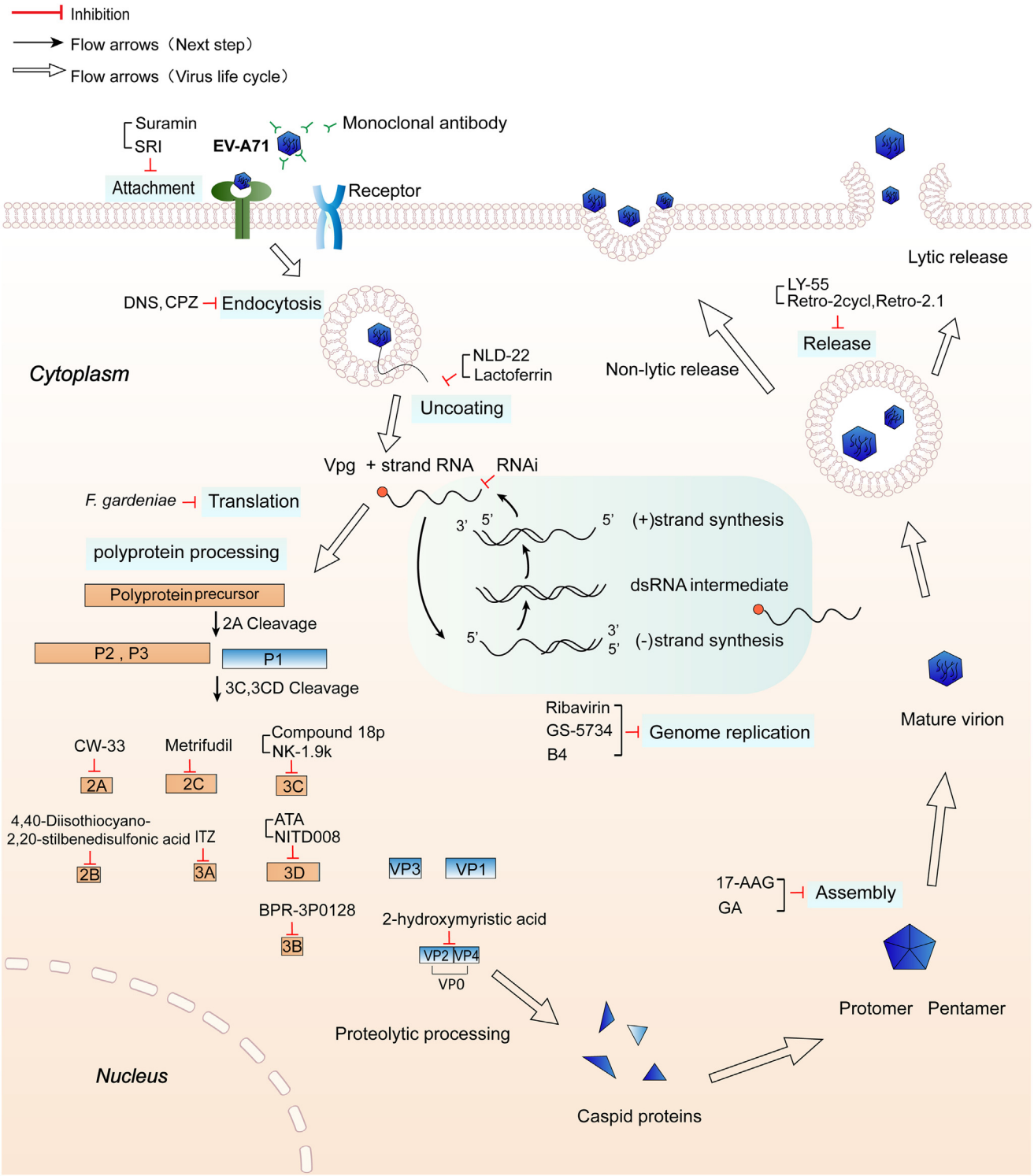

Figure 3.
An overview of the enterovirus life cycle and inhibitors targeting EV-A71 infection. The enterovirus life cycle comprises virus entry (attachment, endocytosis, and uncoating), translation, polyprotein processing, replication, and assembly and release of new virions. If any of these stages is inhibited, the subsequent stages are affected. Representative inhibitors of EV71 are shown in the figure. ATA, aurintricarboxylic acid; CPZ, chlorpromazine; DNS, dynasore; dsRNA, double-stranded RNA; EV-A71, enterovirus A71; GS-5734, remdesivir; SRI, sophoridine; RNAi, RNA interference.

nervous system (CNS). EV-A71 can also be transmitted along the nerve and into the CNS via retrograde axonal transport.

EV-A71 can enter the CNS through 2 routes. The virus can either enter the CNS by the blood—brain barrier or by peripheral nerves through retrograde axonal transport.[17] The EV-A71 virus also transmits to the CNS by peripheral motor nerves, and the skeletal muscle gets immediately infected by the CNS not only by motor neurons but also by other neural pathways.

Factors Related to Viral Infection

The infection of EV-A71 depends on multiple effects of the virus, host, and environment. One of the factors affecting EV-A71 infection is the virulence of the virus. The severity of clinical symptoms caused by EV-A71 varies between genotypes. The change in variation in the sequences in RNA causes some neurologic infections. Li et al[18] suggested that an L97R alteration in the VP1 protein increases the neuronal tropism of EV71, although the alterations in VP1, the 5′ noncoding region (NCR), and protease 2A affect viral virulence.[18,19] Meanwhile, coinfection with EV-A71 and other viruses, such as dengue encephalitis, can affect the severity of viral infections.

Additionally, host susceptibility also influences the virus infection.[20] Some anatomical data show that viral replication in virus-infected patients does not coincide with the site of lesion onset, suggesting that there may be present other pathogenic mechanisms, such as host immunity.[21] One genetic study in Taiwan reported that human leukocyte antigen-A33 is associated with increased susceptibility to EV-A71 infection.[22] It has also been reported that abnormal cytokine activation produces severe inflammation in the lungs.[2] From several studies, it was found that children with severe EV-A71 encephalitis have a cytotoxic T-lymphocyte antigen haplotype (cytotoxic T-lymphocyte—associated protein 4).[23] Currently, tissue-specific viral virulence is poorly understood in cell-based systems and animal models; it needs more study in the future.

In particular, genes involved in mediating EV-A71 virus escape from host intrinsic or adaptive immune response monitoring are closely related to EV-A71 susceptibility.[24] For example, interleukin (IL)-6 and monocyte chemoattractant protein 1 genes may affect the risk and severity of EV-A71 infection by affecting their gene expression and regulating inflammatory response. Most importantly, the level of cytokines fluctuates a lot between healthy volunteers, mild cases, and severe EV-A71-infected HFMD patients with complications, which indicates that cytokines may play a critical role in the progress of EV-A71 infection. Recently, several cytokines or chemokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, multiple ILs , monocyte chemoattractant protein 1, granulocyte colony-stimulating factor, and HMGB1 have been reported to be associated with severe EV-A71 infection.[25]

Pathological Mechanisms of Enterovirus A71 Causing Severe Central Nervous System Diseases

EV-A71 occasionally involves the CNS and induces diverse neurologic complications, such as brainstem encephalitis, aseptic meningitis, and acute flaccid paralysis. Among those complications, brainstem encephalitis is the most critical neurologic manifestation because it can cause neurogenic pulmonary hemorrhage/edema leading to death. Despite the discovery of receptors for EV-A71 in human cells, such as the scavenger receptor B2 and P-selection glycoprotein ligand 1, it is not known why EV-A71 infection predominantly involves the brainstem.[26]

Cytokines, as a part of innate immunity, favor the development of antiviral and type I helper T-lymphocyte immune responses. Cytokines and chemokines play an important role in the pathogenesis of EV-A71 brain stem encephalitis. Both the CNS and the systemic inflammatory responses to infection play important, but distinctly different, roles in the pathogenesis of EV-A71 pulmonary edema.[27]

Endothelin 1, a potent vasoconstrictor, can induce pulmonary edema in rats via intrathecal injections. Endothelin 1 in the CNS may play a role in the development of neurogenic pulmonary edema in children with EV-A71 infection and could be used as a biomarker or therapeutic target for neurogenic pulmonary edema in EV-A71 encephalitis.[28]

However, the mechanisms through which EV-A71 causes neurologic diseases have not been fully explored. Accumulated evidence indicates that EV-A71 infection triggers a plethora of interactive signaling pathways, resulting in host immune evasion and inflammatory response. EV-A71 can activate cellular signaling networks, including multiple cell surface and intracellular receptors, intracellular kinases, calcium flux, and transcription factors that regulate antiviral innate immunity and inflammatory response. Cellular signaling plays a critical role in the regulation of host innate immune and inflammatory pathogenesis.

Histopathologic Characteristics of Enterovirus A71 Infection

Most EV-A71 infections will resolve spontaneously, with a small proportion progressing to severe HFMD. Patients with severe HFMD have inflammatory lesions in several body organs, with the brainstem considered the most vulnerable area. The brainstem and spinal cord are the main target areas in pathological CNS lesions caused by EV-A71.[29] Autopsy and magnetic resonance imaging of patients with EV-A71 brainstem encephalitis showed a widespread inflammatory response in the gray matter of the spinal cord and throughout the medulla oblongata. The pathology suggests extensive vascular dilatation and bruising on the brain's surface, with a high degree of edema in the brain tissue and numerous foci of softening centered on the brainstem and medulla oblongata, satellite phenomena of nerve cells, and the phenomenon of neurophilic cells.[21,27,30,31] Immunohistochemistry staining showed that inflammatory cells in the CNS mainly were CD68+ macrophages/microglia or CD15+ neutrophils, widely distributed in areas with extreme inflammatory changes. EV-A71 may spread from the site of primary infection to the CNS via the inflammatory cell migration pathway.[32]

Neurogenic pulmonary edema is a significant complication of EV-A71 infection and a cause of death. This complication is often secondary to various CNS injuries, such as encephalitis. Pathological analysis showed dilated and congested capillaries in the alveolar walls, widened alveolar septa, massive inflammatory cell infiltration in the interstitium, massive serum exudation in the alveolar cavity, hyaline membrane formation, compensatory emphysematous changes in some lung tissues, and reactive hyperplasia of lymph nodes next to the hilar bronchi. Some investigators found that IL-1B, -6, -10, and -13, TNF-α, and IFN-γ were significantly increased in patients with EV-A71 brainstem encephalitis accompanied by pulmonary edema.[7,20,27]

Pathological observation of EV-A71 infection also reveals a heart with marked focal cardiomyocyte edema and an infiltrate of scattered lymphocytes, a few monocytes, and neutrophils in the intercellular plasm. The spleen showed massive parenchymal cell necrosis or reactive hyperplasia of lymph nodes. In addition, there

was reactive hyperplasia of mesenteric lymph nodes, enlarged lymphoid follicles, and histiocytosis.[21] Although no significant pathological changes were seen in the digestive system and heart in most studies, enlarged tonsils , mesenteric lymph nodes, and varying degrees of inflammatory swelling in the heart were present in some deaths. Based on the pathogenesis and pathological data of EV-A71 infection, we recommend that the treatment of EV-A71 infection observes the principles of early prevention, early recognition, and early treatment, paying attention to its clinical symptoms, improving auxiliary examinations, and the rational use of drugs based on the protection of the body's immune system, thus preventing and treating the CNS and other complications. Additionally, a study found a strong signal of EV-A71 RNA in the epithelial cells of renal tubules.[32,33]

These mechanisms of viral infections and their pathology lay the foundation for their prevention and treatment in the clinical setting.

Antiviral Agents for Enterovirus A71 Infection

The ability of a virus to successfully infect cells depends on its ability to enter and go through a life cycle process (entry, translation, polyprotein processing, replication, assembly, and release of new virions). It means that several steps exist, and each of them represents a target. The key steps of the life cycle provide insights into designing and developing effective antivirals.[34] In the following sections, this review focuses on the inhibitors targeting EV-A71 and highlights examples of each category of inhibitors (Table and Fig. 3).

Inhibitors of Virus Entry

Virus entry is the first step in viral infection, which involves virus attachment, endocytosis, and uncoating. The binding of the virus to the receptor is critical to the tropism of the virus. Known EV-A71 receptors include the human scavenger receptor class B2 (hSCARB2),[35] P-selectin glycoprotein ligand 1 (PSGL-1),[36] heparan sulfate,[37] sialylated glycan,[38] vimentin,[39] nucleolin,[40] annexin II,[41] cyclophilin A (Cyp A),[42] human tryptophanyl aminoacyl-tRNA synthetase,[43] heat shock protein 90 (HSP90),[44] fibronectin,[45] and prohibitin.[46] Some receptors play different roles during viral infection. Among them, hSCARB2, Cyp A, heparan sulfate, sialylated glycan, PSGL-1, and human tryptophanyl aminoacyl-tRNA synthetase are related to virus uncoating. Targeting EV-A71 entry is a promising strategy to inhibit viral infection. Currently, there are many antivirals targeting virus entry.

Virus Attachment

Attachment is the first step of virus entry. The virus binds to specific receptors on the surface of the host, which mediates the process of virus attachment. Blocking adsorption is a very effective method to inhibit viral infection. Some drugs targeting virus attachment are described below.

Wang et al[47] showed that suramin inhibited the early infection of EV-A71 by blocking viral adsorption. Suramin has been used in clinical practice for decades, and its safety is not a concern. Suramin is currently in phase I of the HMFD clinical trial study and it is worth digging into its antiviral activity.[48,49] NF449, a suramin analog, exhibits excellent anti–EV-A71 activity.[50] LJ04 and melittin show outstanding virucidal activity against EV-A71.[51,52] Melittin is currently in phase II of clinical trials for osteochondrosis.[53] Arg-Gly-Asp-Ser can bind to fibronectin and block viral entry. Additionally, SP40 and L-SP40, peptides designed after the VP1 protein of EV-A71, can inhibit viral attachment.[54] Sophoridine and brilliant black BN (E151) prevent EV-A71 infection by inhibiting viral adsorption.[55,56] However, no clinical trial information is available for drugs other than suramin and melittin, and their safety needs further investigation.

Endocytosis and Virus Uncoating

Enteroviruses enter cells via crossing lipid membrane barriers through endocytosis. Then, the virus binds to specific receptors or induces a change in the pH of the cell, thereby helping it to uncoat.[13] Some drugs have been found to inhibit viral entry by targeting endocytosis and uncoating. Endocytosis inhibitors, such as chlorpromazine and dynasore, can inhibit EV-A71 infection.[57] The bovine and human lactoferrin, rosmarinic acid, and NLD-22 protect susceptible hosts by preventing viral uncoating.[58-62] Additionally, compound 11, an inhibitor of Cyp A, can interact with the H-I loop of VP1 to regulate viral uncoating.[42,63] Vapendavir, a novel enteroviral capsid binder, has broad antiviral activity.[64]

VP1, the leading antigen-binding site of EV-A71, is relatively conservative; the development of capsid-binding inhibitors can block the interaction between EV-A71 and the host and improve the drug resistance of EV-A71 mutations.[65] For example, compound 11 may be one of the more clinically promising target compounds for the subsequent development of viral entry inhibitors. Additionally, researchers can enlarge the study of viral infection receptors, which can provide ideas for designing and developing antivirals.

Inhibitors of Viral Translation

When the virus genome enters the cytoplasm, the virus starts the translation phase of its life cycle. The internal ribosome entry site (IRES) is involved in the translation process of the genome. When the translation process is blocked, the viral replication is interrupted. Therefore, the translation stage of the virus is also one of the targets of antiviral design.[66]

Magnesium lithospermate B (MLB) is the main component of Danshen pill injection. Danshen pill injection, a traditional Chinese medicine, was approved in May 2005. MLB can inhibit viral IRES.[67] Quinacrine interrupts RNA transcription and viral protein translation by intercalating into viral nucleic acids.[68] Kaempferol has an anti–EV-A71 effect, and its antiviral mechanism occurs by inhibiting viral translation.[69]

From the point of view of medical application, efficacious inhibitors of viral translation can not only specifically inhibit IRES but also affect the activity of the eukaryotic promoter. As with MLB, it exerts antiviral activity without affecting the action of the eukaryotic promoter in the host. In China, no severe adverse drug reactions have occurred in Danshen pill injection in the past 8 years, so MLB has greater clinical potential among the 3 viral translation inhibitors.[70,71]

Inhibitors of Viral Polyprotein Processing

Enterovirus A71 Structural Proteins

The role of viral structural proteins is mainly to assemble into the viral capsid structure, also known as the capsid protein. Viral

Table
The detailed list and classification of EV-A71 inhibitors: classes, effectivity, test in cell lines and animal models, and clinical trials

No.	Compound	Target	[a]EC_{50}/[b]IC_{50}	Cell line/animal	[c]Clinical trials
Inhibitors of entry					
1	Suramin	Attachment	40 μM	RD	Phase I (HFMD)
2	NF449	Attachment	6.7 μM	RD	-
3	Melittin	Directly inactivates EV-A71	0.76 μg/mL	HeLa	Phase II (tumor-induced osteomalacia)
4	SP40 and L-SP40	Attachment	6-9.3 mM	RD, HeLa, HT-29	-
5	LJ04	Directly inactivates EV-A71	24.3 ± 2.4 μM	MA104	-
6	RGDS	Fibronectin	5 mg/mL, 5 mg/kg	RD, Suckling mice	-
7	SRI	Attachment	31.25 μg/mL	Vero	-
8	E151	VP1	2.39-28.12 μM	RD	-
9	CPZ, DNS	Endocytosis	20 μM, 80μM	HepG2, Vero, RD	Phase III (COVID-19)
10	Lactoferrin	Heparin sulfate glycosaminoglycans	34.5 g/mL	SK-N-SH, RD	Phase II (HIV)
Phase 2 (SARS-CoV-2)					
11	RA	VP1	4.33 ± 0.18 μM	RD	Phase IV (osteoarthritis of the knee)
12	NLD-22	Uncoating	25 pM	RD	-
13	Compound 11	Cyp A	0.37 ± 0.17 μM	Spleen cells	-
14	Vapendavir	Capsid protein	0.7 μM	RD	Phase I (healthy)
Phase 2 (asthma)					
Inhibitors of viral translation					
15	MLB	IRES	0.09 mM	RD	Phase IV (angina)
16	Quinacrine	RNA transcription	9.71 mM	RD	Phase II (prostatic cancer)
17	Kaempferol	IRES	35 μM	RD	Phase I (healthy)
Inhibitors of viral polyprotein processing					
18	LVLQTM	2A	9.6 μM	HeLa	-
19	CW-33	2A	171.2 μM	RD	-
20	4,40-Diisothiocyano-2,20-stilbenedisulfonic acid	2B	1.76 μg/mL	RD	-
21	Adenosine analogs: metridil				
N6-benzyladenosine	2C	1.30 μM			
0.10 mM	RD	-			
22	AN-12-H5	3A	0.55 μM	DLD-1 intestinal	-
23	GW5074	3A	2.00 μM	DLD-1	Phase I (solid tumor)
24	ITZ	3A	1.15 μM	RD	Phase I (RSV/chronic hepatitis B)
25	BPR-3P0128	3B	0.0029 μM	RD	-
26	AG7088	3C	0.014 μM	RD	-
27	Compound 18p	3C	0.030 ± 0.02 μM	RD, Vero	-
28	SG85, PI SG85	3C	180 nM		
0.039-0.200 μM	RD	-			
29	NK-1.8k, NK-1.9k	3C	34.5 nM, 37.00 nM	RD, Vero	-
30	Quercetin	3C	12.1 μM	RD, Vero	Phase III (COVID-19)
Phase 1 (chronic hepatitis C)					
31	NITD008, ppp-NITD008	3D	0.625 μM	RD	-
32	ATA	3D	2.9 μM	RD, Vero	-
33	DTriP-22	3D	0.30 μM	RD, Vero, HeLa	-
34	GPC-N114	3D	0.1-1 μM	RD, Vero	-

Inhibitors of virus replication					
35	Ribavirin	RNA	65 μg/mL	RD	Approved for marketing (broad-spectrum antiviral drugs)
36	Qramine derivatives 4s	Early viral replication	9.1 μg/mL	RD, Vero	-
37	Chebulagic acid	Genome replication	12.5 μg/mL	RD	-
38	Apigenin	Viral RNA transacting factors	10.3 mM	RD, Vero	-
39	GS-5734	Genome replication	0.991 μM	HeLa	Approved for marketing (SARS-CoV2)
Phase 2 (Ebola/HIV)					
40	B4	IFN-β	24.95 ± 0.05 μM	RD, Vero, ICR mice	-
41	Aloe-emodin	ISGFs	0.14-0.52 μg/mL	HL-CZ, TE-671	-
42	CP	I IFN	0.39 ± 0.07 μg/mL	HeLa	-
43	Polyriboinosinic acid (poly(I : C)	IFNs	65 nM	RD	Early phase I (breast cancer)
Phase I (recurrent glioblastoma)					
44	Xiyanping	IFNs	5-10 mg/kg	ICR mice	Approved for marketing (anti-inflammatory/ viral)
45	Minocycline	IL6, G-CSF	100-300 μg/mL	RD, U-87MG, THP-1	Approved for marketing (antibiotics)
46	*Paris polyphylla* Smith	IL-6	78.46 ± 2.80 g/mL	RD	-
47	PD169316	p38	20 μM	RD, HeLa	-
48	Formononetin	ERK, p38, and JNK	3.98 ± 0.80 μM	SK-N-SH	-
49	Resveratrol	IKKα, IKKβ, NF-κB p65	20.2 μM	RD	Approved for marketing (anti-inflammatory/ cancer)
50	Corydaline	JNK, P38, MAPK	25.23 ± 6.60 μM	Vero	-
51	Compound 18	MEK1	10 μM	RD, HEK293	-
52	Berberine	AKT, PI3KIII, MEK/ERK	7.43-10.25 μM	Vero	Phase IV (COVID-19)
Phase III (cirrhosis due to hepatitis B)					
53	GS-9620	PI3K-AKT			
NF-κB	7.43-10.25 μM				
6.0 mg/kg	Vero				
ICR mice	Phase 1 (hepatitis B/C)				
Phase 2 (HIV)					
54	SeNPs@OT	Mitochondrial pathway	9.8-μM SeNPs and 20-nM oseltamivir	U251	-
55	EGCG and GCG	ROS	10 μM	Vero	Phase I (COVID-19)
56	Luteolin	ROS	Approximately 10 μM	Vero, RD	Early phase I (tongue neoplasms carcinoma)
57	Gallic acid	ROS	0.76 μg/mL	Vero	-
58	PDTC	UPS	25-50 μM	Vero	-
Inhibitors of virion assembly and release					
59	Retro-2cycl and Retro-2.1	Viral release	12.56 mM and 0.05 mM	293S	-
60	2-Hydroxymyristic acid	VP0	50 μM	RD	-
61	LY-55	Autophagy	2.22 ± 0.44 μM	Vero	-
62	Anti-FLIP peptide	cFLIP	0-10 μg/mL	MRC5	-
63	GA	HSP90β	2-20 μM	RD, Vero	-
64	17-AAG	HSP90β	0.5-0.2 μg/kg	RD, Vero, hSCARB2-transgenic mice	Phase II (kidney cancer)
Other EV-A71 inhibitors					
65	Raoulic acid	Replication	<0.1 μg/mL	Vero	-
66	Ursolic acid	Replication	0.5 μg/mL	BCC-1/KMC	Phase III (sarcopenia)
67	Glycyrrhizic acid	Event(s) post virus cell entry	3 mM	Vero	Phase IV (immune thrombocytopenia)
68	Corilagin		5.6 μg/mL	Vero	-

(*continued on next page*)

Table (*continued*)

No.	Compound	Target	[a]EC_{50}/[b]IC_{50}	Cell line/animal	[c]Clinical trials
69	Honeysuckle	*let-7a* and viral genome	100-600 nM	RD, SK-N-SH	Phase III (irritable bowel syndrome)
70	mAbs (3A12 , 2A10)	3D	-	Vero , BALB/c ICR mice	-
71	D5, H7, C4	VP1 GH loop	0.203, 0.287, 0.952 μg/mL	RD, Vero, Jurkat T	-
72	mAb51	VP1	10 μg/g	RD, BALB/c mice	-
73	mAbs 22 and 24	VP1	-	RD, BALB/c mice	-
74	JL2	SCARB2	2 μg/mL	293-hSCARB2 cells	-
75	si-2C, si-3C, si-3D	2C, 3C, 3Dpol	-	RD	-
76	si-3′ UTR	3′ UTR	-	RD	-
77	si-5′ UTR	5′ UTR	-	RD	-
78	siRNA-69, siRNA-294, siRNA-319	2A	-	RD	-
79	miR-9	NF-κB	-	RD, Vero, HT-29	
ICR mice	-				

17-AAG, 17-allyamino-17-demethoxygeldanamycin; AG7088, rupintrivir; AKT, protein kinase B; ATA, aurintricarboxylic acid; COVID-19, coronavirus disease 2019; CP, Cortex phellodendri; CPZ, chlorpromazine; Cyp A, cyclophilin A; DNS, dynasore; EGCG, epigallocatechin gallate; ERK, extracellular signal-regulated kinase; EV-A71, enterovirus A71; GA, geldanamycin; GCG, gallocatechin gallate; G-CSF, granulocyte colony-stimulating factor; GS-5734, remdesivir; HFMD, hand, foot, and mouth disease; HIV, human immunodeficiency virus; hSCARB2, human scavenger receptor class B2; HSP90, heat shock protein 90; IFN, interferon; IL, interleukin; IKK, inhibitor of kappa kinase; IRES, internal ribosome entry site; ISFG, IFN-stimulated gene factor; ITZ, itraconazole; JNK, c-jun amino-terminal kinase; mAb, monoclonal antibody; MEK, MAP/ERK kinase; miR, microRNA; MLB, magnesium lithospermate B; NF-κB, nuclear factor kappa B; PI3K, phosphatidylinositol 3-kinase; PI3KIII, phosphatidylinositol 3-kinase, class III; PTDC, pyrrolidine dithiocarbamate; RA, rosmarinic acid; RGDS, Arg-Gly-Asp-Ser; RNAi, RNA interference; ROS, reactive oxygen species; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome–related coronavirus 2; SeNPs, selenium nanoparticles; siRNA, small interfering RNA; SRI, sophoridine; UPS, ubiquitin-proteasome system; UTR, untranslated region.

[a] Concentration for 50% of maximal effect.

[b] Half-maximal inhibitory concentration.

[c] Search website: https://clinicaltrials.gov/ct2/home

antigenic diversity is affected by the structural changes of the capsid proteins VP1 to VP3. Capsid proteins link to viral entry, assembly, and release. Therefore, inhibitors of capsid proteins are classified as inhibitors of viral entry, assembly, and release. It is not explained in detail in this study.

Enterovirus A71 Nonstructural Proteins

2A

The 2A protease has cysteine protease activity and it hydrolyzes the EV-A71 RNA–translated polyprotein into the following 3 consecutive parts: P1, P2, and P3. 2Apro also regulates EV-A71 replication by cleaving 3C and 3D proteins and eukaryotic initiation factor 4GI. Given the properties of EV-A71 2Apro, it has the potential to be an antiviral target.[72] LVLQTM, a pseudosubstrate of 2Apro, can bind to the substrate-binding pocket of EV-A71, interfering with the replication process of EV-A71.[73,74] CW-33 specifically binds to the active site of the viral 2A protease, thereby inhibiting EV-A71 replication.[75]

2B

The 2B protein has viral porin activity and promotes viral transmembrane and genome replication. The 2B protein can also interact with proapoptotic proteins to induce apoptosis and release viral particles. The function of the 2B protein in the virus provides ideas for finding anti–EV-A71 drug targets. Xue et al[76] indicated that EV-A71 2Bpro might mediate chloride-dependent currents in RD cells. 4,40-Diisothiocyano-2,20-stilbenedisulfonic acid can effectively inhibit chloride-dependent currents, thereby preventing EV-A71 infection.

2C

The EV-A71 2C protein is closely related to many critical steps in the viral life cycle, such as viral uncoating and rearrangement of cell membranes. EV-A71 2Cpro also inhibits the phosphorylation of inhibitor of kappa B kinase β (IKKβ), interfering with the nuclear factor kappa B (NF-κB) signaling pathway.[77] N-(2-methylphenyl)methyladenosine and N6-benzyladenosine effectively reduce the probability of host infection with the virus by interacting with 2C.[13,50]

3A

The EV-A71 3A protein is a membrane-bound protein. It can interfere with endoplasmic reticulum division, hinder membrane protein transport function, and promote viral RNA synthesis.[78] Additionally, 3Apro can inhibit the expression of cytokines, such as IL-6 and IL-8, and inhibit the host's antiviral immune function. AN-12-H5 interferes with the early stages of viral infection by targeting EV-A71 3Apro.[79] Itraconazole (ITZ) and GW5074 inhibit the synthesis of EV-A71 new particles by inhibiting 3A. ITZ is currently in phase I of clinical trials of respiratory syncytial virus (RSV). It has greater clinical potential for anti-EV71 development than the other 3A inhibitors.[50,79,80]

3B

The EV-A71 3B protein is also known as the VPg protein. The conserved tyrosine residue at position 3 of the VPg protein forms a phosphodiester bond with the uridine monophosphate at the end of the genome, which can link to the 5′ end of the viral RNA. Then, 3D polymerase–catalyzed VPg uridylation is used as a primer to promote RNA synthesis. Currently, there are relatively few studies on agents targeting 3B.[81] BPR-3P0128, an inhibitor of multiple molecular targeting sites, can inhibit EV-A71 proliferation by targeting EV-A71 VPg uridylation and RNA-dependent RNA polymerase.[82]

3C

The EV-A71 3C protein has multiple functions, such as 3Cpro catalyzes the cleavage of viral precursor proteins and promotes cell apoptosis.[13] 3Cpro can interfere with the polyadenylation of cell RNA by regulating *CstF-64*, thereby enhancing viral infection.[83] Owing to the critical role of 3Cpro in viral replication, it has become a popular target for antiviral research.

Rupintrivir and compound 18p target 3Cpro of EV-A71. The latter has good pharmacokinetic properties in the body.[84,85] Peptidyl aldehyde NK-1.8k and a highly specific α-hydroxynitrile derivative NK-1.9k suppress EV-A71 infection by targeting protease 3C. Compared with NK-1.8k, NK-1.9k has more obvious antiviral properties.[86,87] Additionally, SG85, PI SG85, and quercetin can also inhibit the activity of EV-A71 3Cpro. Among the 3C inhibitors, only quercetin has clinical trial stage information. It is currently in phase III of the COVID-19 clinical trial and has the greatest potential for anti-EV-A71 clinical development.[64,88-90]

3D

The 3D protein, a polyproteinase (RNA-dependent RNA polymerase), uridylylates VPg and uses VPg-pUpU as a primer to initiate virus replication and assists in the elongation of viral RNA strands. 3D is considered an attractive target for drug development.[91] NITD008 inhibits the proliferation of enteroviruses. The triphosphorylated product of NITD008, ppp-NITD008, inhibits EV-A71 infection by inhibiting viral RNA-dependent RNA polymerase activity.[92] Aurintricarboxylic acid and GPC-N114 exhibited inhibitory activity against EV-A71 by interfering with the viral 3D polymerase.[16,93,94] DTriP-22, a novel nonnucleoside analog targeting EV-A71 3Dpol, inhibited EV-A71 replication by reducing the accumulation of RNA.[95] However, the 3D inhibitors have not yet entered clinical studies. There is much research on inhibitors targeting viral proteins, and most drugs focus on 3Cpro. Among them, AG7088 and quercetin have entered clinical trials. For example, a pharmacokinetic-pharmacodynamic study of quercetin in healthy adults and patients with hypercoagulable states has been completed.[96] The advantages of inhibitors targeting viral proteins are more specific and less toxic, but the viral spectrum is narrow, and the risk of developing resistance is high. Combining antivirals with different mechanisms of action not only improves drug efficacy but also reduces the occurrence of resistance. Additionally, most inhibitors of viral polyprotein processing studies have been limited to the cellular level and have yet to be clinically validated. Therefore, investigators can prioritize compounds that have already entered clinical trials as targets for further studies.[97,98]

Inhibitors of Virus Replication

This section introduces the targets of existing anti−EV-A71 replication, including IFNs; ILs; mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and protein kinase B (Akt) signaling pathways; the oxidative stress pathway; the ubiquitin-proteasome system; and so on. Furthermore, it focuses on the inhibitors targeting EV-A71 replication and highlights examples of each category of inhibitors (Fig. 4).

Virus Genome Replication

The core enzyme of genome replication is the viral RNA-dependent RNA polymerase 3Dpol, the replication primer is the viral peptide 3B coupled to 2 uridines, and the viral replication properties are highly conserved. Genome replication is a promising target for researching and designing broad-spectrum antiviral agents.[99]

Ribavirin is an RNA viral mutagen. It exerts broad-spectrum antiviral activity by inducing lethal mutations in the viral genetic material. Ribavirin is one of the most commonly used broad-spectrum antivirals in clinical practice and is susceptible to drug resistance. Currently, the results of clinical trials of phase IV of ribavirin plus pediatric oral solution on HMFD is complete.[100-102] Gramine derivatives 4s and chebulagic acid protect the host by inhibiting RNA replication.[103,104] Apigenin protects the host by disrupting the binding of viral RNA to host transacting factors.[105] Additionally, Ye et al[106] showed that remdesivir (GS-5734)-hindered RNA replication exhibited vigorous antiviral activity against EV-A71. GS-5734 has been approved for the treatment of SARS-CoV2 infections with a favorable safety profile and has high potential for clinical development among these drugs that inhibit EV-A71 genome replication.

Interferons

IFNs are divided into α type, β type, and γ type. IFNs play an essential role in the antiviral defense of cells. IFNs can protect EV-A71-infected hosts by modulating the host's innate immune function and interrupting viral replication.[107,108] Anemoside B4 and aloe-emodin play an antiviral role by upregulating the expression of IFN-β.[109,110] Cortex phellodendri aqueous extract has broad-spectrum antiviral effects by inducing the production of IFNs.[111] Additionally, polyriboinosinic acid acts as a potent IFN inducer that induces the production of type I IFN.[107] Andrographolide sulfonate (Trade name: Xiyanping injection) has inactivating effects on the influenza virus and EV-A71. It can regulate the immune system of host by regulating the secretion of inflammatory factors. Currently, Xiyanping injection is used clinically for antipyretic, anti-inflammatory, and antiviral treatment.[112] Additionally, IFN-α spray or nebulization is effective in the early stages of treating EV-A71 infection.[1,25]

Interleukin

IL is a cytokine secreted by a variety of cells. IL is involved in various physiological and pathological body responses, such as the role of transmitting information and the activation and regulation of the immune system. When a virus infects the body, the host protects itself by producing IL to fight virus invasion.[25] Minocycline reduced EV-A71 offspring production by upregulating IL-6 and granulocyte colony-stimulating factor levels. Minocycline also alleviates CNS complications by reducing TNF levels in the host CNS. Minocycline is approved for marketing as an antibiotic with a proven safety profile. Researchers can continue to study it as an anti-EV-A71 clinical candidate.[113] Additionally, Wang et al[114] found that Paris polyphylla Smith antagonizes EV-A71 replication mainly through the regulation of IL-6 levels.

Mitogen-Activated Protein Kinase

After a virus infects a cell, the toll-like receptor changes its conformation and activates a series of signal transduction, especially MAPK activation. Then, the signal pathways mediated by extracellular signal-regulated kinase, c-jun amino-terminal kinase (JNK), and p38 are activated to start the transcription of various inflammatory factors. MAPK is one of the most studied pathways associated with EV-A71 entry and replication.[115]

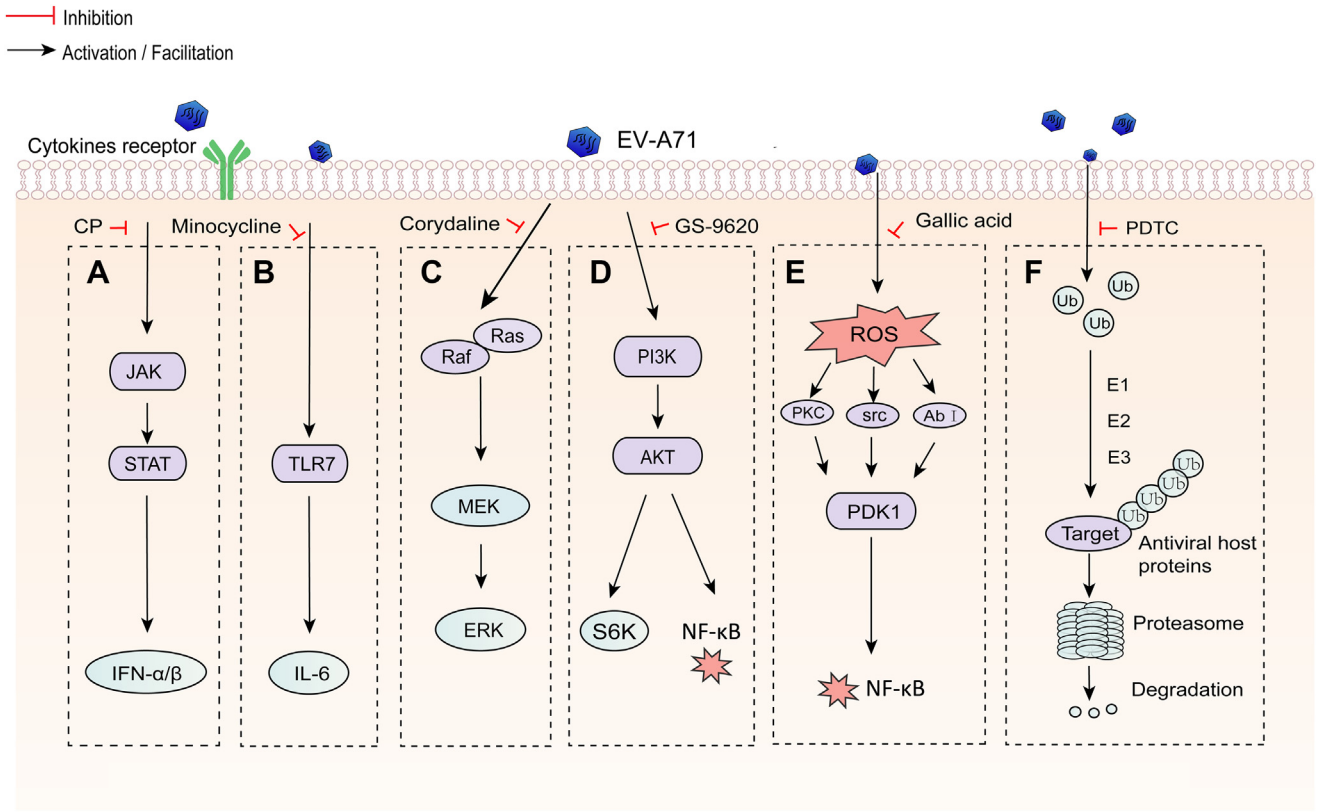

Figure 4.
An overview of the signal pathways and inhibitors targeting EV-A71 infection. Representative inhibitors of EV-A71 are shown in the figure. (A) The interferon pathway. (B) The interleukin pathway. (C) The MAPK pathway. (D) The PI3K/Akt pathway. (E) The oxidative stress pathway. (F) The ubiquitin-proteasome system. Abl, ableson protein tyrosine kinase; Akt, protein kinase B; dsRNA, double-stranded RNA; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; ERK, extracellular regulated kinase; EV-A71, enterovirus A71; IFN-α/β, interferon alpha and interferon beta; IL-6, interleukin 6; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; NF-κB, nuclear factor kappa B; PDK1, 3-phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; S6K, S6 kinase; src, steroid receptor coactivators; STAT, signal transducer and activator of transcription; TLR7, toll-like receptor 7; Ub, ubiquitin.

PD169316, a p38 inhibitor, inhibits cell apoptosis and reduces the release of EV-A71.[116] Formononetin, corydaline, and compound 18 interfere with EV-A71 replication and limit virus release by inhibiting the activation of extracellular signal-regulated kinase, p38, and JNK signaling pathways.[117-119] Resveratrol inhibits the proliferation of EV-A71 by inhibiting the phosphorylation of IKKα, IKKβ, and NF-κB p65 and preventing the secretion of antiviral cytokines. Resveratrol is approved for its anti-inflammatory/anticancer properties and an excellent safety profile and has the potential to be developed into a clinical anti-EV71 drug.[120]

PI3K/Akt

PI3K/Akt is a canonical signaling pathway associated with phosphatidylinositol and plays an essential role in promoting cell survival. This pathway is activated when EV-A71 stimulates cells.[121] Wang et al[122] found that berberine inhibited the phosphorylation of AKT, JNK, and PI3KIII. Later, they also showed that berberine derivatives exhibited significant anti−EV-A71 activity.[123] The heterocyclic compound GS-9620 is classified as a selective TLR7 agonist with potent and low toxicity. It inhibits the dissemination of EV-A71 mainly through NF-κB and PI3K/Akt pathways.[124,125] Furthermore, berberine anti−COVID-19 studies are in phase IV of clinical trials and its safety has been demonstrated. Researchers can prioritize berberine as an anti-EV71 candidate over other PI3K/Akt inhibitors.

Reactive Oxygen Species

Reactive oxygen species (ROS) plays a vital role in host self-defense. ROS is a double-edged sword. When viruses infect cells, they typically turn on their defense mechanisms, producing ROS. When many ROS damage the antioxidant defense system, it will result in the body's oxidative stress response. In some cases, ROS can aid in viral infection.[126]

SeNPs@OT, which are selenium nanoparticles loaded with oseltamivir, reduce EV-A71−induced apoptosis and autophagy of cells through the mitochondrial pathway.[127] Gallic acid, epigallocatechin gallate, and gallocatechin gallate inhibit EV-A71 replication in a concentration-dependent manner. Their antiviral effects are closely related to ROS.[128,129] Luteolin prevents EV-A71 infection by inhibiting the generation of intracellular ROS and delaying the apoptosis of cells.[130,131]

Ubiquitin-Proteasome System

The ubiquitin-proteasome system (UPS) is indispensable in many life activities, such as cell proliferation, differentiation,

apoptosis, and immunity. Meanwhile, it is involved in replicating positive-strand RNA viruses, such as EV-A71 and the dengue virus.[132] The UPS is expected to become a new target for the research and development of antivirals. A study by Lin et al[133] showed that pyrrolidine dithiocarbamate could affect EV-A71 transcription, translation, and viral progeny proliferation by downregulating the UPS. However, there is no clinical trial information for pyrrolidine dithiocarbamate. It needed to be further researched if the drug can be further developed into an anti-EV-A71 drug.

Among the inhibitors of virus replication, several drugs are already in clinical trials. The safety of GS-5734 in hospitalized patients with severe COVID-19 has been studied.[134] In addition, minocycline is a broad-spectrum clinical antibiotic, and its security has not needed to be checked.[135] Given their clinical trial results and significant anti−EV-A71 activity, they could be considered for development as clinical antivirals. Additionally, we may consider a prodrug strategy to guide the development of nucleoside antivirals, considering the vital role that nucleoside analogs, such as ribavirin play as antivirals.[136] The target of these inhibitors is mainly the host. They have the advantage of potentially having a broader spectrum of antiviral activity and less risk of developing resistance but have the disadvantage of off-target effects and cytotoxicity.[71] Therefore, we need to increase the potential for the clinical application of compounds through technological innovation, the search for antiviral-related targets, and improved drug screening and design tools.[137]

Inhibitors of Virion Assembly and Release

The capsid proteins of enteroviruses assemble with newly synthesized genomic RNA to form new infectious mature virions. Some newly generated virions are released extracellularly in vesicles, whereas other virions leave the host via cell apoptosis and lysis. Interestingly, some studies have found that the autophagy of cells can assist the release of viruses in a nonlytic manner.[16,138] When viral assembly and release are blocked, the generation of new viruses is interrupted. Therefore, the viral assembly and release process is a good choice for designing antivirals.

Retro-2cycl and Retro-2.1 block viral release by inhibiting the transport of EV-A71 particles in intracellular vesicles.[139] 2-Hydroxymyristic acid prevents virion maturation by inhibiting the cleavage between VP4 and VP2.[137] The anti-FLIP peptide and lycorine derivative LY-55 prevent the release of EV-A71 progeny by regulating cell autophagy and apoptosis.[140,141] Additionally, geldanamycin, a specific inhibitor of HSP90, reduces the production of EV-A71 progeny by blocking virion assembly. 17-Allyamino-17-demethoxygeldanamycin, an analog of geldanamycin, is consistent with the target of geldanamycin.[134] Data from phase I clinical trials show that 17-allyamino-17-demethoxygeldanamycin can be safely administered in biologically active doses and potentially develop into an anti−EV-A71 drug.[142]

Inhibitors of virion assembly and release belong to the targeted virus drugs. If broad-spectrum antiviral therapy is indiscriminate bombing on the battlefield, then targeted virus therapy is a precise treatment that hits the target. However, the disadvantage of direct-acting viral drugs is prone to drug resistance. Therefore, when screening antivirals, we can pay more attention to medication with a “high-resistance barrier.” It is also possible to try to design a fixed-dose combination of anti−EV-A71 drugs that combines different types/targets of anti−EV-A71 into a single drug, which not only prevents the emergence of drug resistance by inhibiting viral replication at multiple points in the viral life cycle and helps to reduce the burden of multiple doses, but also improves patient compliance. For example, fixed-dose combinations of antiretroviral drugs are very successful cases.[71,136]

Other Enterovirus A71 Inhibitors

Natural Products With Anti-Enterovirus A71 Activity

Many natural products from various plants and animals are widely used in the research against viral infections. Some natural products have shown properties against EV-A71 infection. The advantages of natural products for the treatment of viral infectious diseases are mild action and low cytotoxicity.[143,144] Raoulic acid shows significant anti−EV-A71 activity in Vero cells.[145] Ursolic acid is a natural triterpene carboxylic acid compound with the therapeutic potential to prevent and combat EV-A71 infection.[146,147] Glycyrrhizic acid blocks the replication phase of EV-A71.[148] Corilagin dose-dependently reduces the cytopathic effect induced by EV-A71.[149] Honeysuckle is the dried bud or first-blooming honeysuckle flower with antiviral activity. It attenuates EV-A71 infection by upregulating *let-7a* expression and targeting the viral genome.[150] However, no information on clinical trials of the above natural products against viral infections has been found.

Monoclonal Antibody

After the virus invades the host, cells secrete specific antibodies to neutralize the virus particles, thereby treating viral infection. Monoclonal antibody (mAb) therapy is passive immunity and can effectively prevent infection by specific pathogens. Ideally, mAbs can neutralize not only the antiviral effect of a specific pathogen but also most subgenotypes of a specific pathogen species.[151] A number of mAbs for the treatment of viral infections are currently in clinical development. mAbs (3A12 and 2A10) exhibit significant inhibition of EV-A71 3D polymerase activity.[152] D5, H7, and C4 are mAbs targeting the VP1 GH loop. They do inhibit not only viral entry but also attachment, internalization, uncoating, and RNA release.[153] mAbs 51, 22, and 24 effectively neutralize EV-A71 particles and protect host cells.[154,155] Additionally, JL2 is an mAb that binds to hSCARB2. It plays an important role in protecting target cells from EV-A71 infection.[156]

RNA Interference

RNA interference (RNAi) is an effective means to target specific genes. RNAi therapies are used to treat infections caused by a variety of viruses. RNAi can interfere with EV-A71 genome replication. Small interfering RNAs (siRNAs) that are chemically synthesized can target different parts of the *EV-A71* gene for targeted inhibition. RNAi therapy is currently an effective method used to inhibit EV-A71 activity.[13] si-2C, si-3′ untranslated region (UTR), and si-5′ UTR have an anti−EV-A71 function. They can dose-dependently block the transcription, translation, and replication of the EV-A71 virus.[157,158] siRNA-69, siRNA-294, and siRNA-319 are

3 siRNAs targeting the 2Apro region. They significantly reduce RNA replication.[159] The siRNA targeting 3Cpro and 3Dpol regions can improve survival in a mouse model of infection.[89] Furthermore, microR-9-5p not only modulates the levels of inflammatory factors induced by EV-A71 after attacking cells but also reduces the expression of VP1.[160]

Natural products are abundant. The medicinal effects of some natural products or herbal medicines have been proven after thousands of years of usage. Therefore, the research on antivirals could focus more on those with a convincing track record of use in Chinese medicine. Among them, the multiple targets targeted by natural ingredients are significant advantages for their antiviral effects. mAbs are indicated for patients who have developed resistance to habitual drug use and have used multiple drugs, but viral infection cannot be effectively controlled. RNAi is well targeted, but its delivery system may be potentially toxic in nonclinical toxicology studies in primates.[71,136] We may combine mAbs or RNAi with antivirals to enhance antiviral efficacy and achieve higher functional cure rates. The disease duration caused by EV-A71 infection is short , clinical symptoms are complex, and the effectiveness of drugs in clinical trials needs to be more readily determined, making developing antiviral agents very challenging.[98]

Vaccine

Owing to the lack of effective management and treatment for both mild and fatal EV-A71 infection, vaccination represents the best approach for controlling this life-threatening infection. In December 2015, the China Food and Drug Administration approved the first inactivated EV-A71 whole-virus vaccine for preventing severe HFMD. Different EV-A71 vaccines have been developed, such as inactivated virus vaccines and virus-like particle vaccines, DNA vaccines, subunit vaccines, and live attenuated vaccines.[3] From the previous study, activated whole-virus EV-A71 vaccines showed more effective when compared with other vaccines.[161] Most of the vaccine candidates are still at the preclinical stage of development, and currently a few inactivated EV71 vaccine candidates have completed clinical trials and 3 of them are approved by the China Food and Drug Administration.[162] All vaccine approaches and technologies have advantages and disadvantages, and the conventional EV-A71 vaccines such as live attenuated vaccines were limited by their safety concerns.[163] Modern approaches based on recombinant DNA technology, including recombinant subunit, virus-like particle, epitope-based, DNA, and live vector—based vaccines have been used to address the drawbacks and limitations of conventional vaccines.[163,164] However, improved effectiveness and broader protection against HFMD of these vaccines are needed to be further researched. Similarly to DNA vaccines, they demonstrated very weak and less potent immune responses in larger primates.[11] Hence, further studies on EV71 DNA vaccine candidates need to be conducted to improve their immunogenicity and protective efficacy. Furthermore, there are still a few challenges facing the worldwide use of EV71 vaccines, including the applicability against various EV-A71 pandemic strains in other countries, international requirements on vaccine production and quality control, standardization and harmonization on different pathogen-monitoring and -detecting methods, and so on. Therefore, with EV-A71 vaccines commercially available, there is still a long way to go before reaching effective protection against severe HFMD.

Discussion

HFMD caused by EV-A71 threatens the health of infants and young children worldwide. Although fatal case studies of EV-A71 infection have been reported, the underlying mechanisms of EV-A71 infection, such as how the virus enters the CNS and causes pulmonary edema, have yet to be fully elucidated. To date, there is no approved specific and highly effective antiviral drug for HFMD caused by EV-A71. Therefore, we summarized the pathogenic mechanisms of EV-A71 infection and discussed the recent advances in inhibitors targeting EV-A71 entry, translation, polyprotein processing, replication, assembly, and release of new virions. Additionally, other inhibitors that inhibit EV-A71 through other pathways were also introduced, and the roles of each EV-A71 inhibitor in the virus life cycle were discussed. Although some anti—EV-A71 drugs have been discovered, developing anti—EV-A71 drugs still faces some difficulties, such as drug resistance, off-target effects, and drug safety. With the development of technology and multidisciplinary collaborations, we can gradually solve these problems that limit the research and development of agents by finding more suitable drug targets and constructing reasonable animal models to develop efficient and safe anti—EV-A71 drugs and provide specific and effective protection to children. Additionally, it is hoped that this review will provide guidance and ideas for researchers to develop anti—EV-A71 agents.

Patients with severe HFMD caused by EV-A71 infection are often treated symptomatically. For example, when patients develop intracranial hypertension complications, mannitol is used clinically to lower cranial pressure, and furosemide is added to relieve symptoms when necessary.[165] Blood biochemistry tests revealed elevated blood glucose levels in critically ill patients, and insulin may be applied in cases of severe hyperglycemia.[166] Clinical studies of inflammatory factors in the blood and cerebrospinal fluid of children with HFMD found that the white blood cell count and the level of inflammatory cytokines were significantly elevated in severe cases, and it was proposed that the inflammatory factor storm is one of the mechanisms of brain damage in severely ill children.[3] Therefore, glucocorticoid therapy, such as dexamethasone, is usually applied as appropriate, within 2 to 3 days. Administration of intravenous immunoglobulin and milrinone, a phosphodiesterase inhibitor, has been shown to modulate inflammation, reduce sympathetic overactivity, and improve survival in patients with EV-A71 autonomic nervous system dysregulation and pulmonary edema. Of course, physical adjuvant therapy will be commonly used, such as timely tracheal intubation and the use of positive pressure mechanical ventilation in case of respiratory dysfunction.[167]

EV-A71 has been recognized as highly neurotropic and associated with a diverse range of neurologic diseases, such as aseptic meningitis, brainstem encephalitis, encephalomyelitis, acute flaccid paralysis, and postinfectious neurologic syndromes. The pathogenesis of EV-A71-induced neurologic complications is caused by host-virus interaction, including direct damage by the virus and indirect injury mediated by immune and inflammatory responses.[27] Both innate and adaptive immune mechanisms are important for host defense against viral infections. The production of inflammatory cytokines and chemokines is a unique aspect of the immune responses in the CNS and systemic compartment to EV-A71 infection. Cytokines and chemokines released by EV-A71-infected immune cells may contribute directly or indirectly to disease severity.[25,27] In addition, cellular signaling plays a critical role in the regulation of host innate immune and inflammatory

pathogenesis.[168] Understanding the mechanism of cell signal transduction in regulating host cellular immunity and the occurrence of severe EV-A71 infection will not only help uncover the potential mechanisms of EV-A71 infection–induced pathogenesis but also provide clues for the design of therapeutic strategies against EV-A71 infection.

Given the high preventability of EV-A71 infection, we should actively take control measures to reduce its harm. The EV-A71 inactivated vaccine plays a vital role in the fight against severe HFMD outbreaks caused by EV-A71 infection. However, the coverage of monovalent vaccines is narrow and not universal. Moreover, the development of new combined multivalent vaccines has become complex owing to the diversity of serotypes, high cost, and lack of animal models. So, finding potent anti–EV-A71 drugs combined with vaccines is critical for eradicating EV-A71–associated diseases.

Recently, some drugs targeting EV-A71 have been discovered. Lactoferrin blocked EV-A71 entry by binding to the VP1 region.[58] Quercetin inhibited the activity of EV-A71 3Cpro.[64,90] They both target viruses. Currently, the EV-A71-targeting agents dominate the landscape. However, EV-A71 is an RNA virus, which has posed the problem of resistance owing to the rapid mutations and recombination events of EV-A71. Therefore, drugs with different targets and antiviral mechanisms are needed. The candidates with host-targeting mechanisms have been increasing in recent years. The bioactive compounds that target the host have broad biological and pharmacologic activities. They may have a more comprehensive range of antivirus activity spectrum. The common broad-spectrum antiviral drugs targeting the host are IFNs. Although drugs targeting host cells may have a broader range of antivirus activity spectrum, they may be more toxic to the host and have off-target effects. Therefore, there is an urgent need to develop specific and nontoxic antivirals. Natural products have abundant sources, low prices, stable efficacy, and low toxicity. They have been used extensively by researchers in the development of drugs. Natural products have been widely recognized in the treatment of common infectious diseases, such as influenza, infectious hepatitis, and enterovirus.[144,169] However, the development of anti–EV-A71 agents has been restricted owing to the lack of advanced technology to extract active ingredients from natural products. It is believed that with the deepening of research, natural products will dominate in the field of antiviral treatment.

This review also discussed mAbs and RNAi. mAbs, a type of biological targeted therapy drug, have high specificity, good tolerance, and high safety. However, the purification process of mAbs is complicated, time consuming, labor intensive, and expensive. Moreover, the technologies of large-scale cell culture and animal cell antibody expression are yet to be overcome.[170] RNAi provides a promising antiviral treatment pathway. It can aid in the screening and development of new antivirals and enrich the information library of newly synthesized compounds on their mode of action. However, transferring siRNAs to the appropriate tissues at the appropriate time is a challenge in gene therapy.[171] As we gain more insights into the mechanisms, the knockdown technology might improve vastly with better-designed plasmid- or virus-based vectors.

This review is a summary of the latest progress of existing anti–EV-A71 agents and discoveries, some with the potential for clinical development. Suramin has been used in clinical practice for decades, and its safety is not a concern. Suramin is currently in phase I of the HMFD clinical trial study and its antiviral activity is most worth tapping.[48,49] In China, no severe adverse drug reactions have occurred in Danshen pill injection in the past 8 years, MLB being the main component of Danshen pill injection. So, MLB has greater clinical potential.[70,71] There is a lot of research on inhibitors targeting viral proteins. However, there is no clinical information on most of the drugs. Among them, quercetin is currently in phase III of the COVID-19 clinical trial and has the greatest potential for anti-EV71 clinical development.[89,90] ITZ, a 3A inhibitor, is currently in phase I of clinical trials of RSV. RSV belongs to the same group of RNA viruses as EV71, so ITZ anti-EV71 has some potential for clinical development.[80] Additionally, Ye et al[106] showed that GS-5734 exhibited vigorous antiviral activity against EV-A71. GS-5734 has been approved for the treatment of severe acute respiratory syndrome–related coronavirus 2 infections and has high potential for clinical development among these drugs that inhibit EV-A71 genome replication. Minocycline is approved for marketing as an antibiotic and resveratrol is approved for anti-inflammatory/anticancer properties. They have a proven safety profile and have the potential to be developed into a clinical anti-EV71 drug.[113,120] Furthermore, berberine anti–COVID-19 studies are in phase IV of clinical trials with a proven safety profile. Researchers can prioritize berberine as an anti-EV71 candidate over other PI3K/Akt inhibitors.[122] In addition, many of the inhibitors of virion assembly and release and other EV-A71 inhibitors summarized in this study also have good anti-EV-A71 activity. However, there is a lack of information on clinical trials of these drugs against viral infections.

Researchers have been working hard to study the infection and host defense mechanisms of EV-A71 but have not fully elucidated the details of the EV-A71 transmission process and pathology. Appropriate animal models are needed to understand EV-A71 pathogenesis better and to develop highly effective antivirals. There have been reports of animal models of neonatal mice infected with mouse-adapted EV-A71 strains, cynomolgus monkeys, IFN receptor–deficient mice, transgenic mice with *hSCARB2*, and human *PSGL-1* transgenic mice. However, it is difficult to establish an efficient infection model for EV-A71 owing to ethical, economic, and technical problems. The lack of animal models is also a reason that limits the development of drugs.[172,173] Additionally, drug development is a cumbersome process, a long cycle, and closely related to the application of technology. Approved drugs already have extensive clinical trial data and experience. Therefore, initial studies could focus on previously approved compounds, which can save more cost and time.

Finally, with the rapid development of science and technology and the continuous deepening of research, people will have a deeper understanding and insight into the virus life cycle. Additionally, researchers should closely search for relevant targets of antivirals and improve the methods of drug screening, isolation, and design, and actively carry out interdisciplinary cooperation. Thus, a new chapter is opened for the research and development of anti–EV-A71 drugs.

Author Contributions

Y.W., K.H., and J.W. conceptualized the study. Y.W. and HH.L. wrote and prepared the original draft. Y.W., K.H., and J.W. reviewed, revised and edited the manuscript. K.H. and J.W. supervised the study. Y.W. and K.H. carried out project administration. Y.W., K.H., and J.W. carried out funding acquisition. D.H., Q.H., C.Y., and HL.L. provided comments and suggestions.

Data Availability

Not applicable.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Ethics Approval and Consent to Participate

Not applicable.

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