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REVIEW ARTICLE

The Function and Mechanism of Enterovirus 71 (EV71) 3C Protease

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Abstract
Enterovirus 71 (EV71) is the main pathogen of the hand, foot, and mouth disease. It was firstly isolated from sputum specimens of infants with central nervous system diseases in California in 1969, and has been repeatedly reported in various parts of the world, especially in the Asia-Pacific region. EV71 3C protein is a 183 amino acid cysteine protease that can cleave most structural and non-structural proteins of EV71. Based on the analysis and understanding of EV71 3C protease, it is helpful to study and treat diseases caused by EV71 virus infection. The EV71 3C protease promotes virus replication by cleaving EV71 synthesis or host proteins. Moreover, EV71 3C protease inhibits the innate immune system and causes apoptosis. At present, in order to deal with the damage caused by the EV71, it is urgent to develop antiviral drugs targeting 3C protease. This review will focus on the structure, function, and mechanism of EV71 3C protease.

Introduction

EV71 is a member of the picornaviridae family and is highly infectious in the central nervous system, which causes serious clinical symptoms including encephalitis, poliomyelitis-like paralysis, and even death. However, the pathogenesis of EV71 remains unclear and there is no effective vaccines or drugs to prevent it [1]. The genome of EV71 is a single positive-stranded RNA of approximately 7.4 kb in length and contains an open reading frame (ORF), encoding a polypeptide with 2194 amino acids. The polyprotein is further proteolyzed into P1, P2, and P3 precursor proteins. The P1 precursor protein is proteolyzed into four structural proteins (VP1, VP2, VP3, and VP4). The P2 and P3 precursor proteins are proteolyzed into seven non-structural proteins (2A–2C and 3A–3D) [2]. Among them, the viral protease 3C has been proved to be involved in multiple pathological processes of EV71. EV71 3C protease can cleave the connection site Gln–Gly of P2–P3 [3]. EV71 3C protease can degrade DNA repair enzymes, thereby activating caspase and inducing apoptosis of host cells [4]. EV71 3C protease can inhibit innate immunity by inhibiting type I interferon response [5]. In this article, we will summarize the structure, function, and mechanism of EV71 3C protease.

The Structure of EV71 3C Protease

The crystal structure of EV71 3C protease consists of two similar β-ribbon folded. The long, shallow groove region between the β-ribbon regions is the substrate binding site [6]. The β-ribbon exists in the cleft of the picornaviral 3C protease binding sites to the substrate, and the tip of β-ribbon faces the protease active site. We call this β-ribbon conformation "closed conformation". Unlike picornaviral 3C protease, EV71 3C protease is an open conformation of β-ribbon between βB2 and βC2 (123–133 aa). The β-ribbon flips and the apical end are away from the active site of the EV71 3C protease and are located over the substrate binding cleft [7]. Gly123 and His133 in the β-ribbon form a hinge structure, which plays a key role in the catalytic activity and conformational change of β-ribbon [8]. Previous studies have found that most picornaviral 3C proteases cleave Gln–Gly proteins. Similarly, the catalytic center of EV71 3C protease is Cys–His–Glu [6]. EV71 3C protease contains KFRDI motif (positions 82–86) and VGK motif (positions 154–156) RNA binding domain [3].

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The Role of 3C Protease in the Pathway of Induction of Interferon

Most virus-infected cells produce type I interferons (IFN-α/β) and type III interferons (IFN-λ) or interleukin-28/29 (IL-28/29) [9]. Type II interferons (IFN-γ) are produced only by T cells or NK cells [10, 11]. Picornavirals are mainly recognized by three classical pattern recognition receptors: Toll-like receptors (TLRs), Retinoic acid-induced gene I-like receptors (RLRs), and Nucleotide oligomerization domain-like receptors (NLRs) [12]. We will introduce the role of EV71 3C protease in the three receptor pathways.

The Role of 3C Protease in TLRs Pathway

Studies have shown that TLR1,2,4,5,6 exist on the cell membrane and TLR3,7,8,9,10,11,12,13 exist in the endosomal compartments. TLR4/MD2 (myeloid differentiation) complex, TLR1/6, TLR2 and TLR5 recognize LPS, lipoproteins, and flagellin to activate NF-κB, and induce type I interferon production [13]. TLR7/8 recognizes single-stranded RNA of RNA virus. TLR9 recognizes unmethylated cytosine-phosphate-guanosine (CpG) DNA in bacteria. TLR3 recognizes viral double-stranded RNA and recruits TRIF to induce TRAF3 and activate the TBK1/IKKε complex. Moreover, TRIF also induces TRAF6 to activate the TAK1/TAB2/TAB3/TAB1 complex. After activating TBK1/IKKε, it can phosphorylate IRF7 or IRF3 to induce IFN-I production [14]. After activating TAK1/TAB2/TAB3/TAB1 complex, NF-κB is dimerized and enters into the nucleus, thereby producing proinflammatory cytokines [15] (Fig. 1).

EV71 3C protease can cleave the Q312–S313 site of toll-like ligand TRIF and affect the IFN-β production and NF-κB activation. When the catalytic site H40D of EV71 3C protease is mutated, it cannot inhibit the activation of NF-κB and IFN-β promoters [16]. EV71 3C protease also inhibits NF-κB promoter activation by cleavage of the TAK1 complex by its catalytic activity. EV71 3C protease can cut TAK1 complex, including TAK1Q360-S361, TAB1Q414-G415, Q451-S452, TAB2Q113-S114 and TAB3Q173-G174, and Q343-G344. Overexpression of TAB2 inhibits EV71

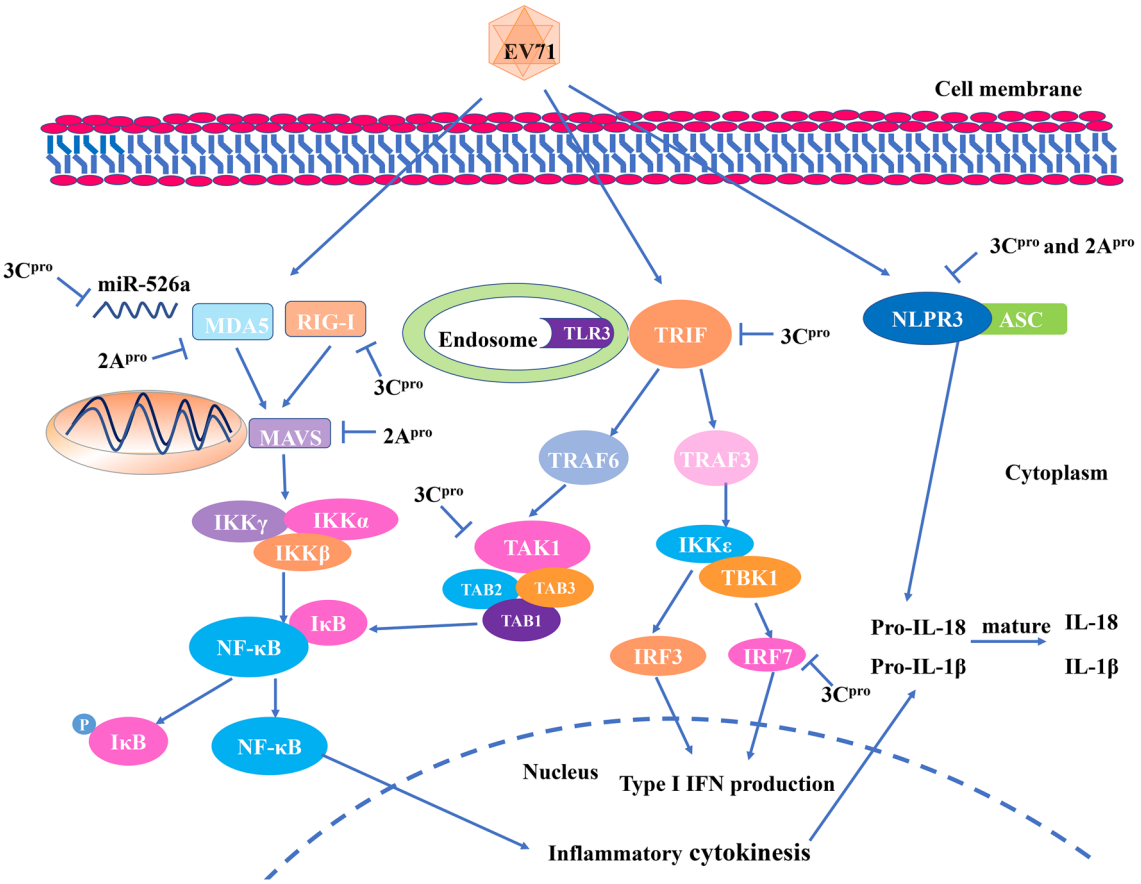

Fig. 1 EV71 3C protease is involved in the mechanism of interferon-inducing pathways. EV71 3C protease, 2A protease is involved in the downregulation of type I IFN and proinflammatory cytokines. In the figure, both EV71 3C protease and 2A protease are labeled to interact with related intracellular signaling molecules

replication. However, fragmentation of TAB2 has no impact on EV71 replication [17]. EV71 3C protease inhibits IRF3 entry into the nucleus and prevents RIG-I pathway from inducing IFN-β production in HT-29 cells [18]. However, another study showed that EV71 3C protease cleaves IRF7 in site of Q189-S190, but had no effect on IRF3 [5]. Furthermore, the 3C protease can cleave purified IRF7, but not IRF3. These inconsistencies have not been clarified [19]. In summary, these studies have shown that EV71 3C protease affects IFN-I production and proinflammatory factor production in part through the TLRs signaling pathway.

The Role of 3C Protease in RLRs Pathway

Members of the RLRs family include RIG-I, MDA5, and LGP2.LGP2 cannot induce type I IFN because it has no CARD structure [20]. RIG-I recognizes double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) virus containing 5′-triphosphate structure.MDA5 mainly recognizes double strand (dsRNA) and positive strand RNA [(+) ssRNA] viruses [21]. RGI-1 and MDA5 have two N-terminal caspase recruitment domains and a C-terminal repressor domain (RD).RIG-I and MDA5 recognize RNA viruses, cause conformational changes, and expose their N-terminal caspase recruitment domains. Subsequently, activated RIG-1 and MDA5 interact with adaptor IFN promoter-stimulating factor 1(MAVS). MAVS binds to IKKα-IKKβ-IKKγ and activates the NF-κB pathway (Fig. 1). In addition, MAVS also activates the TRADD/TANK/TRAF3 complex, thereby activating the TBK1 and IKKε complexes. Activated TBK1 and IKKε complexes can phosphorylate IRF3/IRF7 and induce IFN-I production [22].

EV71 3C protease blocks the binding of RIG-I to IPS-1 and TBK1 by binding to the caspase recruitment domain of RIG-I. EV71 3C protease inhibits IRF3 entry into the nucleus and affected IFN-β production. EV71 3C protease also binds to MDA5, but does not inhibit MDA5-regulated IFN-β production. H40, KFRDI and VDK regions of EV71 3C protease may bind to RIG-I, resulting in inhibition of IFN-β production [5]. EV71-infected cells reduced Lys 63-linked polyubiquitin chains in the N-terminal CARDs region of the RIG-I and inhibit the IFN-I signaling pathway. Increasing the level of ubiquitination of RIG-I can promote the expression of IFN-β and ISGs [23].

Cylindromatosis (CYLD) is a deubiquitinating enzyme that removes Lys 63-linked polyubiquitin chains from RIG-I. CYLD can inhibit the production of IRF3 pathway and IFN-β production [24]. MiR-526a can downregulate the mRNA and protein of CYLD, which increases the expression levels of IFN-I. However, EV71 3C protease downregulated the expression of miR-526a, thereby inhibiting RIG-I regulated interferon type I production [25]. In addition, EV71 encoded-2A protease can cleave MDA5 and MAVS, and then inhibit the IRF3 pathway to mediate IFN-β production [26, 27]. Therefore, EV71 2A protease and 3C protease regulate the activity of MDA5 and RIG-I, thereby affecting the innate immune response.

The Role of 3C Protease in NLRP3 Pathway

In human, there are 22 Nucleotide-binding and oligomerization domains (NOD)-like receptors (NLRs).NLRs recognize pathogens such as bacteria and viruses, and produce activated inflammatory factors such as interleukin-1β (IL-1β) and interleukins-18 (IL-18).Therefore, NLRs can kill or eliminate invading pathogens to maintain the body's immune system balance [28]. The NLRP3 inflammasome is an important component of the NLR inflammatory pathway. Classical NLRP3 inflammasome activation is stimulated by two signals. The first signal activates TLR4 signal pathway, promotes nuclear transcription factor κB (NF-κB) activation and activates the production of precursors such as IL-1β and IL-18. The second signal promotes the assembly of the NLRP3/ASC/pro-caspase-1 protein complex. Pro-caspase-1 self-cuts into an activated form. Activated Caspase-1 also helps pro-IL-1β, pro-IL-18 matures into IL-1β, IL-18 [30] (Fig. 1). The activation of Non-classical NLRP3 inflammasome does not depend on the activation of TLR4 signaling pathway. The latest research shows that caspase-11 directly recognizes intracellular LPS, initiates the activation of NLRP3 inflammasome, promotes the activation and release of gasdermin D, and mediates cell death [29, 31].

After EV71 infection, human primary monocyte-derived macrophages (MDMs) induce proinflammatory cytokines, such as IL-1, IL-6 and tumor necrosis factor a (TNF-a) [32]. Several studies revealed that children with EV71 infection have increased levels of cytokines such as IL-6, IL-10 and IL-13 [33]. Sendai virus and influenza A virus can activate NLRP3 inflammasome [34]. EV71 2A protease specifically cleaves NLRP3 G493-L494. EV71 3C protease specifically cleaves Q225-G226 of NLRP3, but has no cleavage effect on ANL2, ASC, caspase-1, and IL-1β of NLRP3 signaling pathway. Thus, NLRP3 pathway loses regulation of IL-1β and IL-18 production, and does not inhibit EV71 replication. In this review, it is proposed that the patient infection period is divided into 2 stages. Initially, EV71 protein and RNA activates NLRP3 to regulate interleukin production against infection. However, in the later stage, EV71 replicate and produce a large amount of 3C protease and 2A protease. 3C protease and 2A protease specifically cleaves NLRP3, which hinders immune activity [35]. (As shown in Fig. 1, we summarized that EV71 3C protease is involved in the mechanism of interferon-inducing pathways).

The Role of 3C Protease in Interferon Signal Transduction Pathway

Type I IFNs (IFN-α/β) and type II IFN (IFN-γ) are widely expressed in the cell as the first line of defense. These cytokines are capable of combating viral infection and inhibiting virus replication [36]. There are 14 types of type I interferon (IFN-α) in mice, 13 in humans, and one type of β (IFN-β) [37]. Most cells can produce IFNβ. But IFNβ is mainly produced by hematopoietic cells, especially plasma cell-like dendritic cells [38]. The type I interferon (IFN-α/β/ω) receptor is mainly composed of two subunits, IFNAR1 and IFNAR-2. The intracellular domains of IFNAR-1 and IFNAR-2 can bind to Jak1 and Jak2 and activates Jak signaling pathway, resulting in phosphorylation of STAT1 and STAT2. Activated STAT1/STAT2 and IRF9 (p48) form the transcription factor complex interferon-stimulated gene factor 3 (ISGF3). ISGF3 and IFN-stimulated regulatory elements (ISREs) responsible for inducing transcription of related target genes (Fig. 2).

IFN-γ receptor also contains two subunits, IFNGR-1 and IFNGR-2. IFN-γ activates Jak1 and Jak2 pathway and subsequently activates the STAT1/STAT1 complex. STAT1/STAT1 and IRF1 form a homodimer GAF (IFN-γ activating factor) into the nucleus. IFN-γ activating factor combines with GAS elements to induce ISGs transcription [39]. ISGs induce transcription products: including oligoadenylate synthase (OAS), protein kinase R (PKR), and interferon-induced GTP-binding protein Mx (MX) [40, 41]. OAS and PKR can regulate virus replication. RNAase L and MX can inhibit viral transcription [42] (Fig. 2).

3C Protease Affects Type I Interferon-Induced Signaling Pathway

There are a lot of evidence that IFNs have a strong antiviral effect and can be used to treat patients [43, 44]. However, only high concentration of type I IFNs can control EV71 infection and replication. In mice, IFNs pretreatment can avoid infection of EV71. If mice are injected with IFNs after infection with EV71, the antiviral effect of IFNs is small [45]. Studies showed that EV71 reduced the expression of type I interferon receptor IFNAR1, which affects the production of ISGs [46]. However, Liu et al. revealed that EV71 did not significantly downregulate the expression of IFNAR1,

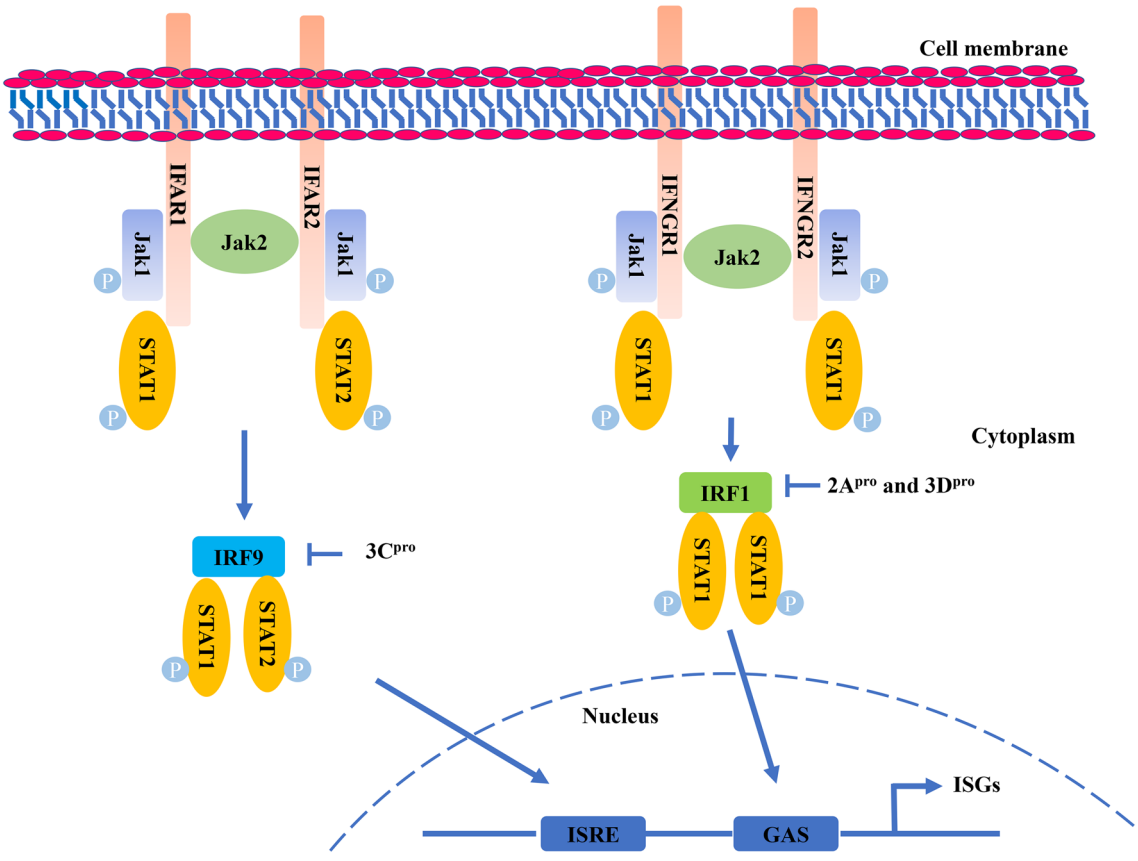

Fig. 2 EV71 3C protease is involved in the interferon signal transduction pathway. EV71 3C protease, 2A protease affects the antiviral effect of the type I IFN signaling pathway. Both EV71 3C protease and 2A protease participate in related intracellular signaling molecule interactions

but downregulate the expression of JAK1 [47]. Wang et al. indicated that EV71 did not downregulate the expression of JAK1 and IFNAR1 [48].

In conclusion, EV71 blocks IFN signaling probably not by downregulating IFNAR1 and JAK1 expression. Hung et al. revealed that the 3C protease of EV71 had a cleaving effect on IRF9, and the C147S point mutation of 3C protease had no cleaving effect on IRF9 [49]. In general, it may be that EV71 3C protease affects the effect of type I interferon by cleaving IRF9, which promotes EV71 replication in the host cells. Thus, it is necessary to further explore the mechanism of EV71 blocking interferon signal transduction.

Effect of 3C Protease on Type II Interferon-Induced Signaling Pathway

Several studies have shown that pulmonary edema and encephalitis resulting from EV71 infection are associated with gene polymorphism and expression of IFN-γ [50, 51]. The mice lacking A129 (IFN-α/β receptor deficient) has a higher mortality rate than AG129(IFN-α/β, γ receptor deficient) [52]. Moreover, the knockdown of the interferon type II receptor and STAT1 in mice increased the EV71 lethality [53]. It indicates that type II interferon may also play an important role in antiviral activity.

Wang et al. showed that EV71 2A protease and 3D protease prevented IFN-γ induction of IRF1 activation, resulting in failure of STAT1 phosphate nucleation [54]. Therefore, EV71 attenuates IRF1 activation in the IFN-γ signaling pathway and promotes replication in the host cells (as shown in Fig. 2, we summarized that EV71 3C protease is involved in the interferon signal transduction pathway).

The Role of EV71 3C Protease in Host Cell Apoptosis

Picornaviral 3C protease can activate caspase and induce apoptosis of host cells. EV71 3C protease triggers cell DNA degradation and apoptotic bodies [55]. Li et al. reveals that the 3C protease can degrade poly (ADP-ribose) polymerase, a DNA repair enzyme. This study showed that 3C protease can activate caspase and induce apoptosis in SF268 cells. Caspase inhibitors (DEVD-fmk and VAD-fmk) can block apoptosis caused by 3C protease [4]. It has been reported that EV71 3C protease affects the polyadenylation of host mRNA by cleaving CstF64 [56]. EV71 3C protease relies on its protease activity to cleave GSDMD, a significant component of pyroptosis, resulting in cell pyroptosis [57]. Li et al. demonstrated that EV71 3C protease promotes apoptosis through cleaving pinx1, a telomere binding protein. What's more, 3C protease cleaves the pinx1at the site of Q50-G51 pair, and accelerates EV71 release [58]. Chen et al. recently confirmed that SUMO E2-conjugating enzyme Ubc9 promotes ubiquitination of EV71 3C protease, which helps to reduce EV71 replication and cell apoptosis [59]. The ubiquitination of EV-A71 3C protease inhibits viral replication and host cell apoptosis.

In addition, endoplasmic reticulum-associated degradation (ERAD) component p97 can participate in EV71 replication. EV71 3C protease induced Ubc6e cleavage may be a key mechanism for EV71 to inhibit ERAD [60]. In conclusion, EV71 3C protease facilitates EV71 replication by affecting endoplasmic reticulum molecules.

EV71 3C Protease Inhibitors and Potential Treatment for EV71 Infection

The 3C protease play important roles in EV71 replication and apoptosis of host cells. Thus, the development of antiviral drugs targeting 3C protease has become a hot spot. EV71 3C protease has no homology with mammalian proteases, so 3C proteases can be used as antiviral targets. According to the specificity of 3C protease structure, anti-EV71 drugs were designed, such as inhibitors of 3–5 amino acids and aldehyde peptides, which can form irreversible covalent bonds with the 3C protease active site.

Broad-Spectrum Inhibitors of 3C Protease

Rupintrivir (AG7088) has extensive antiviral activity against HRV and a variety of enteroviruses. EV71 3C protease catalytic structural amino acid residues (His40, Glu71 and Cys147) are strictly conserved. Rupintrivir can covalently bind to the active site of EV71 3C protease. Studies have shown that this binding mode is relatively stable. Yeast two-hybrid cell experiments also demonstrated that Rupintrivir effectively binds to EV71 3C protease. Besides, Ruprintrivir does not reduce the frequency of the common cold, but has a significant antiviral effect in the common cold [61].

Modified Peptide Inhibitor of 3C Protease

Based on the covalent combination of Rupintrivir and 3C protease, a series of highly inhibitory drugs have been developed, such as substance-based peptidomimetics, aldehydes and derivatives. Kuo et al. found that Rupintrivir enhanced antiviral activity by replacing the unsaturated ester with the aldehyde at the P1' position [7].The EV71 3C protease inhibitor, pseudopeptide aldehyde 5×, has strong inhibitory activity and low cytotoxicity [62]. Wang et al. reported that Peptide NK-1.8k inhibits EV71 3C protease activity and EV71 proliferation [63]. α-Keto amide exhibits good inhibition of 3C protease activity and relatively low cytotoxicity, so it can be used as an inhibitor of EV71 3C protease [64].

And the experimental results show that if the inhibitor has a rotatable ester bond, it can enhance the interaction of 3C protease at the substrate binding site of S4 and increase the antiviral activity [65].

Natural Medicine and Other Inhibitors

Hung et al. found that Rupintrivir and interferon have a synergistic effect on the inhibition of EV71. Fisetin and rutin have been reported to inhibit EV71 3C protease with an IC50 value of 85 μM and 110 μM, respectively [66]. CPI (Diisopropyl chrysin-7-yl phosphate) can bind toEV71 3C protease and inhibits its activity. The main component of the CPI, chrysin, can be extracted from natural flavonoids in many plants [67]. Due to the excellent inhibitory activity of Cyanohydrin on 3C protease, Cyanohydrin derivatives can be used as EV71 3C protease inhibitors [68]. Adenosine analog (NITD008) inhibits EV71 replication. Its EC50 value is 0.67 mM, but it has relatively high cytotoxicity (CC50 1/4 119.97 mM) [69].It is reported that EV71 3C protease inhibitor DC07090 exhibited the inhibition potency with an IC50 value of 21.72 ± 0.95 mM without apparent toxicity ($CC50 > 200$ mM) [70].

Conclusion

EV71 is the leading cause of severe hand, foot and mouth disease, which is a serious threat to the world, especially in the Asia-Pacific region. Studies have shown that EV71 3C protease plays important roles in its pathogenesis. The research on EV71 3C protease has made great progress. The crystal structure of 3C protease has been determined, the catalytic active site and RNA binding site have been found. The innate immune system, as the body's first line of defense, plays key roles in the fight against microbial infections, especially the type I interferon response. EV71 attenuates interferon signaling, allowing EV71 to evade immune mechanisms. However, the EV71 escaping mechanism has not been fully elucidated. The 2A and 3C protease of EV71 have cleavage inhibition on some important linker molecules. Research on anti-EV71 3C protease drugs has made some progress. The role of EV71 in the pathogenic mechanism still needs further study. The targeting medicines against 3C protease are still needed to be further explored.

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Author Contributions Weihui Wen and Zixuan Qi drafted the manuscript together and Jing Wang was responsible for conception and design.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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