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Antiviral peptides against *Enterovirus A71* causing hand, foot and mouth disease

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ABSTRACT

The emergence of new and resistant viruses is a serious global burden. Conventional antiviral therapy with small molecules has led to the development of resistant mutants. In the case of hand, foot and mouth disease (HFMD), the absence of a US-FDA approved vaccine calls for urgent need to develop an antiviral that could serve as a safe, potent and robust therapy against the neurovirulent *Enterovirus A71* (*EV-A71*). Natural peptides such as lactoferrin, melittin and synthetic peptides such as SP40, RGDS and LVLQTM have been studied against *EV-A71* and have shown promising results as potent antivirals in pre-clinical studies. Peptides are considered safe, efficacious and pose fewer chances of resistance. Poor pharmacokinetic features of peptides can be overcome by the use of chemical modifications to improve *in vivo* delivery particularly by oral route. The use of nanotechnology can remarkably assist in the oral delivery of peptides and enhance stability *in vivo*. This can greatly increase patient compliance and make it more attractive as antiviral therapy.

1. Introduction

The emergence and re-emergence of viral pathogens capable of causing epidemics or pandemics pose urgent attention to the research community to develop novel vaccines and antivirals. The development of effective and safe vaccines against novel emerging pathogens is especially challenging and time-consuming. In the recent pandemic of coronavirus disease (COVID-19) caused by *severe acute respiratory syndrome coronavirus 2* (*SARS-CoV-2*), it is estimated that it will take from 18 months to two years to develop an effective vaccine with an efficacy of at least 50 % [1]. This is the shortest time frame envisaged for the development of an effective and safe vaccine at pandemic speed to prevent further escalations of the contagion, unlike the normal research process for vaccine development which may take up to 10 years. An alternative to prevent or treat viral infections is to focus on the development of antiviral drugs which, in the absence of an effective vaccine, could be valuable to prevent or treat the infections.

Enterovirus A71 (*EV-A71*) is one of the main etiological agents of hand, foot and mouth disease (HMFD). Other HFMD pathogens that have caused major outbreaks include *Coxsackievirus-A16* (*CV-A16*), *Coxsackievirus-A10* (*CV-A10*) and *Coxsackievirus-A6* (*CV-A6*). However, *EV-A71* has been proven to be a neurotropic virus capable of causing severe neurological syndromes with high fatalities in Asia [2]. To date, there is no US-FDA Food and Drug Administration (FDA) approved vaccine or antiviral therapy for neurovirulent *EV-A71* causing HFMD. The FDA has approved nearly 100 antiviral drugs for different viral infections over the past 50 years. More than half of these antivirals are used to treat *human immunodeficiency virus-1* (*HIV-1*), *influenza virus*, *herpes simplex virus* (*HSV*) and *hepatitis B* and *C viruses* [3].

2. Development of antivirals

2.1. Molecules from natural sources as antivirals

Antivirals can be isolated from natural sources such as plants, microorganisms, mammals, arthropods and marine organisms. These antivirals have made their way to clinical therapeutics such as podophyllotoxin for perianal and genital warts, oseltamivir for influenza virus and tenofovir for *hepatitis B virus* (*HBV*) and *HIV* infections [4,5]. Vidarabine is another FDA-approved antiviral that interferes with viral DNA replication but was discontinued in 2001 due to solubility and rapid degradation issues [3]. Oxymetrin from *Sophora flavescens* was shown to be a promising antiviral agent against coxsackievirus B3 induced myocarditis in mice [6]. Moreover, there are many flavonoids

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with varied antiviral mechanisms of action that have been recently reviewed [7]. However, the isolation and purification of natural antiviral compounds from plants or mammals is a long, laborious, time-consuming and expensive process [8].

2.2. Small molecules as antivirals

Small molecules have made tremendous advancement in the antiviral field in recent years. Most of the small molecules can enter the cell conveniently and elicit downstream interactions due to their small size. Small molecules can modify protein functions and alter protein-protein interactions. Many small molecules have been developed to treat viral infections. Some of these have been successfully used to treat viral infections while others are undergoing pre-clinical and clinical trials.

Recently approved small molecules by FDA (from 2018 onwards) for viral infections include tecovirimat for smallpox, baloxavir marboxil for influenza viruses and doravirine and bictegravir for HIV-1 and HIV-2 infections, respectively (www.fda.gov). Pimodivir is a small molecule that is in phase III clinical trials for influenza infections [9]. Likewise, for HIV, many small molecules such as LA Cabotegravir, Elsulfavirine and Fostemsavir are currently under phase III clinical trials [10–12].

Apart from off-target toxicities owing to adverse drug effects to patients, the major problem associated with small molecules is resistance posed by viruses. Therefore, there is a need to identify antiviral agents that are more specifically targeted, less susceptible to resistance and with fewer side effects. For an antiviral to be useful and has limited side effects, its target and mechanism of action must be thoroughly understood. The ideal target will be a viral process that is essential for the replication of the virus so that it cannot mutate to form escape mutants. If the virus will mutate at crucial positions, it will have to compromise its fitness and therefore, its survival.

2.3. Peptides as antivirals

Peptides are now widely accepted as drugs and there is an increasing number of peptides that are being tested as antimicrobial agents in clinical trials [13]. Table 1 lists peptides that have reached clinical development for their antiviral potential against specific viral pathogens and their mechanisms of action.

Peptides can be divided into several groups based on their net charge, hydrophobicity, helicity, or structure. Antiviral peptides are cationic and possess amphipathic characteristics which enable them to be designed as therapeutic peptides [18]. For the treatment of viral infections, peptides can be an ideal candidate. Designed antiviral peptides against viruses are usually derived from the sequence of the virus and it could inhibit interactions with the host component, hence preventing entry or fusion of the virus [19,20]. Apart from selectivity, peptides are less susceptible to resistance because different peptides might target multiple functional targets of viruses and different stages of the viral life cycle. Selecting and combining antiviral peptides that could inhibit different stages of the virus cycle will be advantageous. Commercially, only one peptide – Enfuvirtide is marketed as an antiviral against HIV and it could suppress viral entry into the cells by inhibition of fusion of the virus with the host cells [21].

2.3.1. Advantages of peptides

The main advantages of peptides over small chemical compounds are specificity, tolerability, potency, rarer side effects (as the final breakdown products are amino-acids) and commercial scalability. Moreover, peptides have the potential to interact at the active site of large proteins where protein-protein interaction is essential. Lead identification has been much easier now with advancements in structural and genomic technologies. However, the short half-life, solubility, bioavailability, stability as well as delivery of natural peptides are major challenges faced by peptides. These could be overcome by physicochemical and structural alterations during formulation development to make peptides more acceptable as therapeutics and are discussed in section 5 of the review. The delivery of antiviral drugs has been improved by nanocarriers. In addition, nanoparticles have led to enhanced bioavailability and modified pharmacokinetics [22].

FDA has approved many peptides that are currently being used as therapeutics. For example, vancomycin is generally prescribed in clinical practice to treat resistant bacterial infections. Bacitracin and neosporin are known peptides given to infants to treat pneumonia and other staphylococcal infections by external applications [23].

Although small molecules have made great medicinal progression in the preceding century, it has brought along with it the uncertainties of resistance. In the case of antivirals, the efficiency of small molecules has been repeatedly hampered by the emergence of resistant mutants. These mutated viruses pose dangers of re-emergence of viral infections that could become a global challenge. Influenza virus - a causative agent of seasonal flu, has frequently mutated over the years and rendered many anti-influenza drugs futile. For example, resistance towards antivirals such as amantadine, rimantadine, zanamivir and oseltamivir has emerged [24]. Moreover, relatively newer approved antivirals for *influenzavirus* such as baloxavir marboxil (Xofluza) and peramivir

Table 1
Antiviral peptides in clinical trials.

Name	Mechanism of action	Virus	Status	Sequence	Identifier	Reference
Myrcludex B	Sodium taurocholate co-transporting polypeptide (NTCP) inhibitor (Entry inhibitor)	*Hepatitis B virus*	Clinical phase II trial	Myristoyl-GTNLSVPNPLGFFPDHQLDPAFGANSNNPDWDFNPNKDHWPEANKVG	NCT02637999	[14]
Hepalatide (L47)	Surface antigen (HBsAg) blockers	*Hepatitis B virus*	Clinical phase I trial	Undisclosed	NCT02612506	[15]
Adaptavir	C-C chemokine receptor type 5 CCR5 receptor antagonist	*Human immunodeficiency virus*	Clinical phase II trial	ASTTTNYT	NCT00951743	[16]
Aviptadil	Inhibitor of intraleukin-6, tissue necrotic factorα and N-methyl-D-aspartate -induced caspase 3 activation	*Severe acute respiratory syndrome coronavirus 2*	Clinical phase II trial	HSDAVFTDNYTRLRKQMAVKKYLNSILN	NCT04311697	[17]

(Rapivab) have to be used with caution to avoid resistance.

Another instance where resistance to small molecules has hampered the development of antivirals is in the case of the *respiratory syncytial virus*(*RSV*). Virus escape mutants were soon identified in tissue cultures and the general population against small molecules (GS-5806, RV-521 and JNJ-53718678) due to viral mutations such as P488I/V, D486 N, D498Y and L141W. Some of these mutations contributed to reduced fitness of the virus while others helped to increase or maintain the viral fitness through stabilizing the F protein [25–27]. In comparison, peptide candidates such as 3ac against respiratory syncytial virus targeted the 6B complex to inhibit the post-fusion stage. Therefore, it was reported that the antiviral activity of the 3ac peptide would not be diminished against *RSV* escape mutants [28].

Likewise, antivirals developed against *EV-A71* have faced great challenges of resistance. A test compound ribavirin was reported to induce resistant mutants in cultures after several passages of the virus in its presence. Mutations such as S264 L, G64R, G64 T in the 3D polymerase were identified as the prime reason for reduced susceptibility of *EV-A71* towards ribavirin [29]. Similarly, I113 M and V123I mutations in the VP1 gene were detected upon serial passaging of the virus in the presence of new capsid binding pyridyl imidazolidinone-based small molecules (NLD and ALD) [30].

One of the solutions to avoid resistance against *EV-A71* is to switch the inhibitory target from viruses to the host. The relatively shorter half-life of the peptide could be favourably exploited by using small peptides that could bind to receptors used by viruses for entry into the cells. The peptide would degrade faster than chemical molecules and leave the biological system without harmful toxic compounds that are usually generated by small molecules. Tan et al. (2012) identified the SP40 peptide that could potentially inhibit the receptor(s) involved in the entry of *EV-A71* into the host cell [31]. However, the exact receptor involved is currently under investigation. He et al. (2018) also identified a small peptide (RGDS) that blocked the fibronectin receptor and inhibited the viral entry of *EV-A71* [32]. Nonetheless, these peptides are required to be further evaluated and modified to improve the stability and systemic bioavailability before they could be proposed as antiviral peptides for clinical use.

2.3.2. Limitations of peptides

Peptides appear to be potential antiviral drugs but they have to overcome some obstacles. One of the main hurdles associated with peptides is the high cost of production. Using short peptides such as RGDS, an antiviral peptide against *EV-A71*, would be more favourable to reduce the cost of production, Moreover, identification of minimal residues necessary for antiviral activity could be achieved by evaluating the truncated peptides of an identified antiviral peptide. Newer methods of peptide synthesis and purification methods of amides as well as production from recombinant peptide expression could be exciting approaches to mass-produce the antiviral peptides.

Apart from the cost of production, some of the major limitations associated with peptides are stability, bioavailability, short half-life and mode of delivery. Peptides are poor candidates to cross the physiological barrier [33]. Hence, therapeutic peptides are usually administered *via* subcutaneous, intramuscular or even intravenous routes to overcome the poor ability to cross the physiological barrier. A well-known example is an antiviral peptide, Enfuvirtide. The anti-HIV peptide is required to be injected twice daily to keep the viral loads under control. The high frequency of injections and increased cost (approximately USD 90 per day) result in decreased patient compliance with the recommended antiviral therapy. An alternative route to overcome the bioavailability of peptides is the transbuccal route of administration. The transbuccal route had been reported to combine the use of gold nanoparticles patented by Midatech [34] and PharmaFilm™ (MonoSol Rx) technology in the delivery of peptides.

In general, a drug must have at least 20 % of oral bioavailability in the body. However, peptides can hardly achieve 1% bioavailability, making them a poor candidate as oral therapeutics. Poor bioavailability in combination with a shorter half-life makes peptide concentrations to be unavailable in systemic circulation for pharmacological effects. For example, the short half-life of ~4 h of Enfuvitide could be overcome by chemical modifications that could pave the way for the development of future antiviral peptides as effective therapeutic agents [35]. Many factors can contribute to the instability of peptides in the physiological system. Enzymes such as hydrolases, proteases and peptidases are ubiquitously present in the body. When a peptide enters the biological system, it could rapidly be degraded by these enzymes. Moreover, physical barriers in the mucosal membrane, endocytosis and efflux pumps could greatly impact the bioavailability of peptides [36].

Apart from peptides being given intravenously, all other routes of administrations could lead to rapid metabolism by enzymes at the site of administration when injected subcutaneously, intramuscularly or through the first-pass metabolism in the liver when administered by oral routes. This results in a sharp decline in the concentration of peptide drugs in the body, leading to poor pharmacodynamics profile of therapeutic peptide [37]. Some of these limitations could be overcome by using strategies such as chemical modifications and nanomaterials which are discussed later in section 5.

3. Antiviral peptides against non-*EV-A71* enteroviruses

Therapeutic peptides were traditionally derived from plants and animals. With the advancement of technology, peptides responsible to protect humans from various microorganisms were identified/isolated from screening recombinant/peptide array libraries or those synthesised *de novo* [34]. The *de novo* peptides could also be found against a specific target by docking peptide libraries or rationally synthesising peptides using structural biology-based approaches. Several antiviral peptides isolated from natural resources have been documented against diverse groups of viruses [38–40]. Similarly, many peptides have been demonstrated to exhibit antiviral properties against enteroviruses [41,42]. Antivirals against *EV-A71* are discussed in section 4.

3.1. Peptides from animal/insect origin

Alloferon (HGVSGHGQHGVHG), a well-characterised peptide isolated from the blowfly, and its derivatives were evaluated for their antiviral activities against *CoxsackievirusB2* 971 PT and a clinical isolate of *Coxsackievirus B2*. It was identified that substitution of histidine at position 1 with lysine (Lys1-alloferon) could drastically improve the activity of alloferon and it was able to confer replication inhibition against CV-B2 971 PT and its clinical isolate [38]. However, the off-target effects of alloferon on other immune responses could be disadvantageous. Another naturally occurring peptide, melittin, was identified from the honeybee (*Apis mellifera*) venom and was reported to inhibit the Coxsackievirus H3 infection *in vitro*. The inhibitory mechanism was found to be virucidal [43]. Similarly, natural antimicrobial peptides produced by the normal flora of human gut lining such as human β-defensin 3 (hBD3) was found to be up-regulated during picornavirus infections, indicating a natural extracellular antiviral response. It was reported that poliovirus-1, *CV-A16* and *CV-B5* infections were inhibited significantly by hBD3 *in vitro* [44]. This is consistent with earlier reports of mouse β-defensin 3 that was identified to protect HeLa cells *in vitro* and myocarditis in mice from *CV-B3* infection [45].

3.2. Milk protein peptides

Milk proteins and peptides have been widely studied for their antiviral activities against several viruses. One of the extensively studied proteins is lactoferrin and a small peptide lactoferricin derived from the N-terminal region of lactoferrin. Lactoferrin binds either to the structural proteins of viruses or to the receptor(s) of host cells [46]. In both circumstances, it could prevent viral attachment and/or internalisation.

Marchetti et al. (1999) reported that lactoferrin has the potential to affect *poliovirus* replication *in vitro*. Many lactoferrin derivatives including apo-lactoferrin, iron, zinc and manganese saturated lactoferrins were able to prevent the entry of poliovirus in Vero cells [47]. It was interesting to note that not human lactoferrin but bovine lactoferrin was able to provide relatively better antiviral effects against poliovirus [48]. In another study by Tinari et al. (2005), bovine lactoferrin was found to inhibit the replication of echoviruses by blocking viral interaction with the receptor and prevented apoptosis in the cells infected with echoviruses [39]. The exact mechanism for lactoferricin is unclear for enteroviruses. However, it was proposed to interfere with host cell microtubules and reduced intergrase nuclear distribution in the case of *HSV-1* and *HIV-1*, respectively [49,50].

3.3. Synthetic peptides

Proteolytic cleavage inhibitor peptides play an important role in the reduction of viral infections. These peptides with few amino acids and electrophilic anchors mimic the substrate and increase the likelihood of being selected by the proteolytic enzyme instead of the natural substrate. Such peptide inhibitors include Micheal acceptor derivatives and peptide aldehydes [51]. For example, Magsoudi et al. (2010) reported a peptide inhibitor against 2A protease ($2A^{pro}$) of *CV-B3*. They designed a 16-mer synthetic peptide (GRTTLSTRGPPRGGPG) that could compete with the natural substrate for $2A^{pro}$ at the active site. This resulted in the prevention of host cell apoptosis, suggesting that this peptide worked as anti-*CV-B3* [52]. Most of the antiviral peptides have been shown to have the potential to inhibit enteroviruses *in vitro* and should be further explored as therapeutic agents *in vitro*. A few examples of antiviral peptides against enteroviruses are listed in Table 2.

4. Antiviral peptides against *EV-A71*

Viruses causing HMFD generally infect children under the age of 6 years. Approximately 2 million HMFD infections are reported in China each year and *EV-A71* accounts for close to a million cases. Most of the infections usually result in mild symptoms such as fever with rashes and ulcers in the mouth. However, infections with virulent strains of *EV-A71* were reported to cause severe neurological symptoms, leading to reduced cognitive ability, acute flaccid paralysis and death [53]. Many small molecules, siRNA, antibodies and natural products have been evaluated for antiviral activities against neurotropic *EV-A71*. However, none has progressed to clinical trials due to reasons like the emergence of resistant mutants or limited efficacy of antivirals across multiple *EV-A71* genotypes/sub-genotypes. This poses a need to continue the search for antiviral molecules that could provide inhibitory activities against various genotypes/subgenotypes of *EV-A71*. Since peptides are usually designed to target against structural proteins (conserved regions) of viruses or against the receptors used by viruses to enter the host cell, these could be investigated as antiviral molecules against *EV-A71*. Current literature has limited information in terms of the research involving peptides as antivirals against *EV-A71*.

4.1. Lactoferrin from milk protein

The first report of the peptide exhibiting antiviral property against *EV-A71* in rhabdomyosarcoma (RD) cells was reported by Lin et al. (2002) [54]. Lactoferrin inhibited various strains of *EV-A71*, probably by blocking the host cell receptors (glycosaminoglycans – heparin sulfate) as well as the VP1 structural protein of *EV-A71*. Moreover, lactoferrin was able to induce IFN-α and reduced virus-induced IL-6 production in SK-N-SH cells. It must be noted that only the preincubation of cells with lactoferrin was able to confer antiviral activity. The longer the preincubation time, the better the antiviral activity was observed *in vitro* [54].

When tested in the murine model, lactoferrin on its own conferred 30 % protection when seven-day-old mice were challenged with *EV-A71* [46]. It was interesting to note that when the porcine lactoferrin protein was expressed in transgenic mother mice, 100 % of the pups were able to survive *EV-A71* lethal challenge on the seventh day since birth. However, when pups were challenged on the second day and fourth day of birth, the lethality was 50 % and 33.3 %, respectively. The viral loads were significantly reduced as shown by the reduction of viral RNA copies determined by RT-PCR which validated the earlier reports that oral lactoferrin could protect the suckling pups from *EV-A71* infection [55]. The comparison of both studies suggested that when 5 mg lactoferrin was directly administered to young mice, the lower protection observed was probably due to insufficient lactoferrin. When lactoferrin was continuously expressed from mother mice harboring the porcine lactoferrin transgene, the quantity of lactoferrin present in the milk was significantly higher. This enhanced production of lactoferrin could confer the higher protection being observed.

4.2. Melittin peptide from bee venom

Bee venom has been widely studied for the presence of several antimicrobial peptides including mast cell degranulating peptide, apamin, melittin and adolapin. When bee venom was evaluated by Uddin et al. (2016) for the antiviral activity against *EV-A71 in vitro*, it was found that bee venom could exert highly potent antiviral effects at as low as 2.0 μg/mL concentration. To identify the active component involved in antiviral activity, melittin, a 26 amino acid long peptide (GIGAVLKVLTTGLPALISWIKRKRQQ), was tested in HeLa cells. Melittin inhibited *EV-A71* infection by a direct virucidal effect. The virus was co-incubated with melittin for 30 min which resulted in a marked reduction of virus-induced cytopathic effect 24 h post-infection. Moreover, a 4-fold

Table 2
Antiviral peptides against non-*EV-A71* Enteroviruses.

Peptide	Sequence	Enterovirus	Model	Suggested Mechanism	Reference
Alloferon and its analogues	HGVSGHGQHGVHG	971 PT *Coxsackievirus type B2* (*CV-B2*) and several clinical isolates	*In vitro*	Induction of interferon by nuclear factor kappa B (NFκB) signaling pathway	[38]
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	*CV-H3*	*In vitro*	Virucidal action	[43]
Human β defensin 3 (hBD-3)	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK	*Poliovirus-1*, *CV-A16* and *CV-B5*	*In vitro*	Extracellular antiviral response/ virucidal action	[44]
Lactoferrin	MKLFVPALLSL……..STSPLLEACAFLTR	*Poliovirus*	*In vitro*	Inhibition of viral attachment or internalisation into the host cells	[47]
–	GRTTLSTRGPPRGGPG	*CV-B3*	*In vitro*	Inhibition of replication by blocking 2A protease	[52]
SP40	QMRRKVELFTYMRFD	*Poliovirus 1*, *CV-A16*	*In vitro*	Inhibition of attachment	[31]

Complete sequence of lactoferrin has been provided in table footnotes.

reduction in mRNA levels of viral protein 1 (VP1) was observed when compared to the untreated virus. It was interesting to note that the highest concentration of melittin used in these experiments was only 2.0 μg/mL, indicating that the peptide was highly potent. However, melittin was found to be very toxic to cells at low concentrations as it could cause 50 % cell death at 4.36 μg/mL [43].

4.3. Host defensive peptides

Host defensive peptides are expressed by upregulation of pro-inflammatory cytokines which is a classic immune response when toll-like receptors interact with pathogens. However, not many peptides were known to be upregulated during *EV-A71* infection. Chen et al. (2018) recently found that a naturally occurring 45 amino acid long human β-defensin 3 (hBD3) was up-regulated during picornavirus infections, particularly by *EV-A71*. To identify if hBD3 has a role in the inhibition of the virus and the mechanism of inhibition, *EV-A71* was pretreated with recombinant hBD3 protein extracellularly or recombinant hBD3 was transfected into the cells to study intracellular effects. Only recombinant hBD3 proteins but not intracellularly expressed hBD3 in colon adenocarcinoma intestinal cells (HT-29 cells) were able to confer inhibitory effects, confirming that inhibitory effects were extracellularly mediated. This suggested that hBD3 had the ability to block the entry of *EV-A71* into the host cells during the early phase of infection to provide protection from infection [44].

4.4. Synthetic peptides

4.4.1. SP40 peptide

Tan et al. (2012) screened 95 (15-mer) synthetic peptides spanning over the entire VP1 protein (297 amino acids) of *EV-A71*. Four peptides (SP40, SP45, SP81 and SP82) were identified to significantly inhibit *EV-A71* infection (>80 %) *in vitro* in rhabdomyosarcoma cells. Peptide SP40 (amino acid residue 118–132 in the VP1) had the highest antiviral activity amongst all 95 peptides used in the screening. SP40 was further investigated as an antiviral peptide with IC_{50} of 6–9 μM *in vitro*. The peptide was also non-cytotoxic to the cells when treated up to the concentration of 280 μM. The amino acid sequence of SP40 has been identified to be conserved in several genotypes/subgenotypes of *EV-A71*. Moreover, SP40 was also able to inhibit *CV-A16* and *poliovirus* infections *in vitro*, making it a broad spectrum anti-enteroviral peptide that could be used for prevention or treatment of hand, foot and mouth disease as well as poliomyelitis.

An alanine scanning method was used to further elucidate the residues that were critical for antiviral effects. Positively charged amino acids in the sequence of the SP40 peptide were found to be important for inhibition of *EV-A71*. Particularly, arginine at position 3 was able to confer significant biological activity of the SP40 peptide. It was identified that SP40 peptide could inhibit *EV-A71* when cells were pre-treated with the peptide for 1 h prior to infection and it blocked the attachment or entry of *EV-A71* into the cells. Receptors that have been discovered to facilitate attachment and/or entry of *EV-A71* include scavenger receptor class B - member 2 (SCARB-2), P-selectin glycoprotein ligand-1 (PSGL-1), sialylated glycans, annexin 2, heparan sulfate, vimentin, cyclophilin A, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), galectin-1, nucleolin, fibronectin, prohibitin, and human tryptophanyl-tRNA synthetase (hWARS) [56]. Since the mode of action of viral inhibition exerted by the SP40 peptide was identified as cell protection (prophylactic), it has been postulated that SP40 could interact with any of the receptor(s) involved in the attachment or entry of *EV-A71* into the cells. However, the exact receptor(s) with which SP40 interacted to inhibit *EV-A71* infection is yet to be identified [31].

4.4.2. G2 peptide

Peptides G1 and G2 were previously shown to reduce the *HSV* infection by inhibition of heparan sulfate, an anchoring molecule that aids in the attachment of viruses to host cells [57]. Tan et al. (2013) investigated anti-heparan sulfate peptides G1 and G2 against *EV-A71*. When rhabdomyosarcoma cells were pre-incubated with peptides for 1 h before *EV-A71* infection, peptide G2 but not G1, was able to inhibit *EV-A71* infection by up to 76.5 % at 1000 μg/mL, as determined by plaque assay and qRT-PCR [58]. However, the concentration at which this inhibition was achieved is far too high and therefore, it might require significant chemical modifications before it could be further considered as a potential candidate.

4.4.3. LVLQTM peptide

A short peptide, LVLQTM, was previously shown to inhibit human rhinovirus replication by acting as a pseudosubstrate of 2A protease. Since both human rhinovirus and *EV-A71* belong to the *picornaviridae* family, the peptide was evaluated for antiviral activity against *EV-A71*. LVLQTM acted as a pseudosubstrate of $2A^{pro}$ and affected the replication of *EV-A71* by inhibiting the cleavage of eukaryotic translation initiation factor 4 G (eIF4G) in HeLa cells. It was interesting to note that more potent inhibitory effects were observed when the peptide was added 4 h after infection as it produced a 417-fold reduction in viral titres. The molecular interaction studies between the $2A^{pro}$ and LVLQTM peptide suggested that strong hydrogen bonding and Van der Waals forces resulted in conformational changes of protease. Thus binding of LVLQTM to the substrate-binding pocket of *EV-A71* had prevented virus replication. The amino acid sequence of the $2A^{pro}$ is highly similar to the $2A^{pro}$ in 40 strains of *EV-A71*, in particular, the Asn129 residue is highly conserved and serves as an important interaction site. Therefore, this peptide was proposed to be used as a broad-spectrum antiviral against multiple strains of *EV-A71* [59].

4.4.4. Anti-FLIP peptide

A FLICE-like inhibitory protein (FLIP) is part of the death effector domain (DED) family that did not exert direct actions, rather it regulated autophagy, signaling of NF-κB and apoptosis by binding to other members of the DED family [60]. Lee et al. (2009) found a 10-mer anti-FLIP peptide (EVVLFLLNVF) that inhibited the regular functions of the FLIP protein. The viral FLIP (vFLIP) was recognised to play an important role in the pathogenesis of *EV-A71* by interfering with the signaling pathway of apoptosis. Won et al. (2012) evaluated this anti-FLIP peptide against *EV-A71* and found that the peptide was able to inhibit cytopathic effects (CPE) caused by *EV-A71* in MCR5 (fibroblast) cells. When cells were preincubated with the 10 μg/mL peptide, autophagy was markedly induced and caused disruption in the function of FLIP, which resulted in the inhibition of viral replication [60]. It was interesting to find that not only antiviral-FLIP but also anticellular-FLIP was able to render marked antiviral activity. They also identified that the most critical amino acid residues Leu-Phe-Leu (LFL) within the functional domain of the peptide that were involved in protein-protein interactions for antiviral activity.

4.4.5. Anti VPg uridylylation peptide

The process of viral replication generally requires priming to start the replication of the genome. In the case of *EV-A71*, a small peptide (VPg or 3B) is used as a primer for this purpose. The primer undergoes the process of uridylylation, where uridine residue is added to the molecule. VPg is required to be uridylylated before it could bind to 3D polymerase of *EV-A71* for replication. Lou et al. (2014) reported that inhibition of VPg uridylylation could halt the replication of *EV-A71*. A small peptide of ten amino acids was found to inhibit the process of uridylylation and it was proposed as a potential antiviral candidate against *EV-A71* [61]. Conversely, very limited information is available in the literature regarding this peptide and thus necessities further in-depth study.

4.4.6. RGDS peptide

He et al. (2018) identified a synthetic peptide, Arg-Gly-Asp-Ser

(RGDS), as an inhibitor of the fibronectin receptor which was found to facilitate entry of *EV-A71* into the cells. Pretreatment of rhabdomyosarcoma cells with the RGDS peptide (5 mg/mL) for 9 h led to around 80 % inhibition of the virus. The RGDS peptide was able to block fibronectin (FN) which is a receptor for attachment of *EV-A71*. The peptide bound to fibronectin and interrupted viral entry in cell cultures which exhibited reduced infectivity. Moreover, the efficacy of this peptide was evaluated in suckling mice. The peptide was found to confer 75 % survival of newborn mice up to day 13 due to decreased viral loads. Moreover, marked improvement was observed in body weights and clinical scores of RGDS treated pups [32]. However, the concentration of RGDS peptide for treatment at 5 mg/kg in mice was high and this could pose a problem for clinical application.

An understanding of the molecular mechanisms involved in various stages of *EV-A71* infection could lead to the rational design of antivirals. The molecules that have been shown to modulate virus pathogenesis or inhibiting virus entry by competing with cell attachment factors for binding to *EV-A71* could be ideal candidates. Antiviral peptides known to inhibit *EV-A71* are presented in Table 3.

5. Therapeutic applications of peptides

5.1. Chemical modifications to overcome limitations

Short plasma half-life and poor oral bioavailability are limitations facing the applications of antiviral peptides. Peptides are prone to degradation by the enzymes present in the gastrointestinal tract. Proteases secreted from the pancreas (chymotrypsin, trypsin, carboxypeptidase) can cause 20 % degradation of these peptides. Another barrier is the absorption of peptides from the epithelial layer of the gastrointestinal tract due to the presence of mucin gel, glycocalyx and glycoproteins. Moreover, maximum peptide degradation is observed in brush borders where microvilli help in nutrient absorption from food. Peptide molecules that succeed in passing through these barriers can also encounter efflux pumps that may reverse their flow back into the intestine. Presystemic first-pass metabolism through portal vein and liver is yet another challenge for oral delivery of peptides which decreases bioavailability. Even potent peptide drugs given orally will have short half-lives (usually in minutes) due to rapid clearance from the system. This may result in the necessity for increased frequency of dosage and lead to reduced patient compliance. Currently, only 9 peptides are marketed in the oral dosage form (Table 4) but there is none against viral infections [62].

The biological activity of peptides can be retained or improved with alterations of their physicochemical properties. Approaches such as the delivery of enzyme inhibitors along with peptides, carrier systems, absorption enhancers, chemical and structural modifications (peptidomimetics) have been reported previously [63]. Natural amino acids are in the L- configuration. Modification of amino acids in L-peptides to its D-enantiomers can lead to resistance to enzymatic degradation and prolonged systemic half-life [64]. Moreover, D-enantiomers have been reported to possess very low to no immunogenicity with high potency [64]. Jaishankar et al. (2015) reported a novel D-peptide DG2 (D-MPRRRRIRRRQK) that targeted *HSV-1* entry was able to suppress *HSV* infection. The DG2 peptide was found to be very stable and successfully resisted degradation by proteases during tryptic digestion for up to 60 min due to the modification of all L-amino acids to D-isomers in the sequence [65]. The use of antivirals with D-isomers is relatively new and needs to be tested for its serum stability, metabolism and elimination in physiological systems *in vivo*.

Retro-inverso peptide is another way of modification from L- to D-conformation by flipping the peptide sequence from the C to N terminal region [66]. Levi et al. (2004) identified a peptide from the *HIV* antibody representing the specific complementarity-determining region. This peptide was entirely changed to non-natural D-amino acids and retro-inversed to reduce the susceptibility to enzymatic degradation. This peptide showed greater inhibition than its L-peptide [67].

Another approach to protecting a peptide from enzymatic degradation is cyclisation. It was demonstrated to increase the half-life of a peptide by Werle et al. (2006) [68]. Cyclisation can be achieved by different kinds of chemical bridging such as biaryl, disulphide ether bridges [63,69]. Peptides were derived from complementarity-determining region loops present in broad-spectrum antibodies against various influenza virus strains. The design used cyclisation and non-proteogenic amino acids to improve the pharmacokinetic and therapeutic spectrum of antiviral peptides [70]. However, it must be kept in mind that cyclisation could make peptide structure rigid and might render it inactive in some cases [63].

Table 4
Marketed peptides in oral dosage form.

Peptide	Trade Name(s)	Company	Treatment
Colistin sulphate	Koolistin®	Biocon Ltd. (India)	Bacterial infections
Cyclosporine	Neoral® Sandimmune®	Novartis AG (Switzerland)	Immunosuppression
Desmopressin acetate hydrate	DDAVP® Tablets DDAVP® Melt Minrin®	Ferring Pharmaceuticals (Switzerland)/ Generic (e.g. Actavis Labs FL Inc., NJ, USA)	Central diabetes insipidus, primary nocturnal enuresis
Glutathione	Reduced L-Glutathione	Theranaturals Inc. (ID, USA)	AIDS-related cachexia/cystic fibrosis
Linaclotide	Linaclotide	Acatavis, Inc. (NJ, USA) /Ironwood Pharma, Inc. (MA, USA)	Irritable bowel syndrome, chronic idiopathic constipation
Rimegepant	Nurtec ODT	Biohaven Pharmaceuticals	Acute migraine
Taltirelin hydrate	Ceredist® Ceredist OD®	Mitsubishi Tanabe Pharma Corporation (Japan)	Spinocerebellar degeneration
Tyrothricin	Several brands	Several	Pharyngitis
Vancomycin HCl	Vancocin®	ANI Pharmaceuticals, Inc. (MN, USA)	Bacterial infections

Adapted from Aguirre et al. (2016) [62].

Table 3
Antiviral peptides against *EV-A71*.

Peptide	Sequence	*EV-A71* strain (genotype/subgenotype)	Antiviral Activity/IC_{50}	Cytotoxicity/CC_{50}	References
Lactoferrin	MKLFVPALLSL……..STSPLLEACAFLTR	Strains 2272, 1743, 1470 and 13,091	10.5 – 24.5 μM (bovine) 103.3–185.0 μM (human)	NR	[48,54]
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	NR	0.76 ± 0.03 μg/mL	4.36 ± 0.54 μg/mL	[43]
SP40	QMRRKVELFTYMRFD	A, B and C	6 – 9.3 μM	>280 μM	[31]
G2	MPRRRRIRRRQK	B4	%I = 76.5 %	NR	[58]
LVLQTM	LVLQTM	Accession number AEF32490	Dissociation constant of 9.6 μM	NR	[59]
RGDS	RGDS	Accession number JN230523.1	NR	NR	[32]
Anti-FLIP	EVVLFLLNVF	C Accession number JN544418	5.9 ± 0.2 μg/mL	NR	[60]

Complete sequence of lactoferrin has been provided in table footnotes. N.R = Not reported, % I = Percentage inhibition of virus.

Addition of PEG (molecule with hydrophilic and hydrophobic part) aids to attain dual solubility and stability to degradation by proteolytic enzymes dues to steric hindrance. Better systemic stability of PEGylated molecule and enhanced absorption was reported by Suk et al. [71]. Lately, Wang et al. (2019) used PEGylation to improve the pharmacokinetic parameters of the C34 peptide which was marketed as the antiviral peptide Enfuvirtide (T20). They showed that PEG not only provided improved half-life from 1.1 h to 5.1 h but it had further increased the affinity of the peptide C34 towards its target (*HIV-1* peptide N36). Moreover, the flexibility of PEG can be exploited to make a spacer molecule. Recently, Xia et al. (2020) used a PEG molecule as a flexible spacer to anchor cholesterol with EK1 peptide to enhance the activity against SARS-CoV-2 and other Coronaviruses. This dual-use of PEG and cholesterol further strengthened the capacity of the peptide to inhibit the growth of coronaviruses *in vitro* and *in vivo* [72].

Similarly, protein lipidization was found to confer stability and thus prolonged half-life. This is due to the facilitation of transport of the peptides through membranes. Reduced peptide bonds, pseudo peptides, nitrogen substitution at the α-carbon in the backbone are some of the other ways towards producing superior oral peptides [63].

Stapled peptides are α-helical peptides with linking residues on the backbone of hydrocarbon chain usually at position i and i + 3, i + 4 or i + 7. These linking residues are olefinic amino acids (UAA) and do not hamper the interactions between peptides and targets [24]. Stapling has been demonstrated to improve plasma half-life owing to resistance to proteases *in vitro* and *in vivo* [73].

5.2. Oral administration of peptides

Currently, most peptides are administered as injectables and the challenges facing oral administration include acidic and enzymatic degradation in the gastrointestinal tract as well as their ability to cross the intestinal mucosa. Chemical modifications of peptides for oral administration include stapling of peptides, building hydrophobic patches, cyclisation, amidation or acetylation, methylation and establishment of hydrogen bonds [36].

To facilitate the oral delivery of peptides, many carriers have been studied. It may or may not be accompanied by absorption enhancers or enzyme inhibitors. Thiomers, mucoadhesive polymers, emulsion systems, liposomes, hydrogels and nanoparticles are a few examples. It is very important to consider the size of the final formulation as a bulky carrier system can hinder the entry of peptides through the mucosa owing to poor absorption and bioavailability. An ideal system would involve peptides that are small in size that can penetrate the cell, resist enzymatic degradation and release the active molecule by simple dissociation. Drug delivery systems like Eligen® containing absorption enhancers such as SNAC [sodium N-8-(2-hydroxybenzoyl) amino caprylate] could be complexed with the peptide to render a lipophilic system which could internalise and dissociate in cells to release drug-molecule in its active form for pharmacological activity [74]. SNAC has been used to deliver peptides *in vitro* and by the oral route in the animal model. B12™ is a marketed product that has used SNAC as an oral delivery agent [75]. Insulin and GLP-1 analogues have also been reported to use conjugation with SNAC to formulate the oral dosage form and they have reached clinical trial phase I [76]. Peptelligence®, Robotic pill®, Nanocells® are other technology platforms currently in use by pharmaceutical industries to boost oral delivery of peptides [77]. Similarly, lipid-based nano-suspensions are becoming popular delivery agents for *in vitro* and *in vivo* administration and have been studied extensively to develop an improved carrier system for clinical settings [78].

The cell membrane is a natural selective barrier preventing the entry of peptides into the cell. Cell-penetrating peptides (CPPs) which are positively charged short peptides (5–30 amino acids) have been shown to penetrate the biomembrane and delivering peptides for intracellular functions, both *in vitro* and *in vivo*. CPPs could enter the cells directly by transduction through the membrane (pore formation or membrane destabilisation) or undergo endocytosis (including micropinocytosis) [79]. CPP shuttles could be chemically synthesised or be driven from known proteins (for example, the TAT protein). Many CPPs are currently undergoing clinical trials for evaluation to transport peptide cargoes against various diseases [80].

5.3. Potential translation of nano carrier-based antiviral peptides to clinical application

With the success of Enfuvirtide as an antiviral peptide, there is a great potential that other peptide drugs can enter the clinical setting. However, the challenges associated with drug delivery require attention. The nanomaterials that could enhance the oral delivery of peptide-based drugs have been reviewed [81]. Enhanced antiviral effects could be brought about through the use of conventional enzyme inhibitors, chelators, protease inhibitors encapsulated in nanomaterials to the most recently reported biodegradable microneedle drug cargoes. Nanoparticles made of polymeric particles such as lactic acid, ester amides and fatty acid esters provide hydrophobic interactions, thus allowing increased retention time at the mucosal lining. Inorganic particles such as gold, silica and selenium provided better drug release profiles when compared to polymeric particles due to their ability to withstand the acidic environment.

The factors in successful oral peptide delivery may also involve diameter, surface charge and surface modifications of nanoparticles. Moreover, peptide ligand modification, loading with CPP or RGDS peptide and nanocrystalisation have been proposed as some of the ways to improve the penetration, stability and long-term retention of biological activity [82]. Nanocrystallisation or nanosuspension of the pure therapeutic drug has the advantage of 100 % drug delivery. Nanoformulations also allow masking the taste of drugs and therefore, can quickly be adapted for drugs that require long term usage for specific groups of population such as the paediatric and geriatric patients to increase the compliance rate of therapy [22]. Nanoparticles can affect the pharmacokinetics, pharmacodynamics and therapeutic characteristics of encapsulated drugs along with improved safety [82]. Nanoparticles can also be considered as an option to address the challenges of manufacturing thermolabile or easily degradable peptides and thus allow nanodrugs to move towards clinical applications [22].

6. Conclusion

Many antiviral candidates including small molecules, antibodies and natural compounds have been discontinued for clinical development due to the increasing emergence of resistant mutants. Peptide therapeutics can be an alternative approach to overcome the emergence of resistance by targeting essential processes of pathogenesis or entry into the host cells. Peptides have an advantage over many small molecule drugs as the latter could only interact with a limited area of the target and this interaction could not mimic the selective binding of a peptide with the host cell receptor. Moreover, peptides have the capability to interact with a larger area at an active site of the host cells that require protein-protein interactions. The limitations of peptides such as poor bioavailability, permeability, short half-life and high cost of production can be overcome by using chemical modifications and nanotechnology. Moreover, nanocarrier-mediated delivery to enhance oral bioavailability, as well as stability of peptides in biological systems can further warrant the development of effective peptide therapeutics. Recent literature has shown promising results using advanced technologies both *in vitro* and *in vivo* systems. However, further clinical studies are needed to identify the antiviral peptides that can be translated in the clinical setting.

In the case of developing antivirals against *EV-A71*, many small molecules that have been studied in pre-clinical stages have posed the problem of resistance due to the rapid mutations of *EV-A71* against the

test compounds. Peptides that have been studied as antivirals against *EV-A71* mostly affect attachment of the virus either by targeting host receptors or the viral protein. Out of all the peptides identified in virto, only a few peptides have been evaluated *in vivo* against *EV-A71*. Therefore, there is a definite need to further study these peptides *in vivo* and apply chemical modifications for the development of more stable therapeutic antiviral peptides against *EV-A71*.

Declaration of Competing Interest

The authors declare no conflict of interest.

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