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Antiviral Peptides: Identification and Validation

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Abstract
Despite rapid advances in the human healthcare, the infection caused by certain viruses results in high morbidity and mortality accentuate the importance for development of new antivirals. The existing antiviral drugs are limited, due to their inadequate response, increased rate of resistance and several adverse side effects. Therefore, one of the newly emerging field "peptide-based therapeutics" against viruses is being explored and seems promising. Over the last few years, a lot of scientific effort has been made for the identification of novel and potential peptide-based therapeutics using various advanced technologies. Consequently, there are more than 60 approved peptide drugs available for sale in the market of United States, Europe, Japan, and some Asian countries. Moreover, the number of peptide drugs undergoing the clinical trials is rising gradually year by year. The peptide-based antiviral therapeutics have been approved for the Human immunodeficiency virus (HIV), Influenza virus and Hepatitis virus (B and C). This review enlightens the various peptide sources and the different approaches that have contributed to the search of potential antiviral peptides. These include computational approaches, natural and biological sources (library based high throughput screening) for the identification of lead peptide molecules against their target. Further the applications of few advanced techniques based on combinatorial chemistry and molecular biology have been illustrated to measure the binding parameters such as affinity and kinetics of the screened interacting partners. The employment of these advanced techniques can contribute to investigate antiviral peptide therapeutics for emerging infections.

Keywords Binding evaluation techniques · High throughput screening methods · In silico approaches · Peptide based therapeutics

Introduction

The infections caused by viral pathogens including clinical viruses or naturally emerging viruses pose a serious threat worldwide. Unfortunately, only few therapeutics are available for limited viruses like Human immunodeficiency virus (HIV), Hepatitis virus, Herpes simplex virus (HSV) and Influenza virus (Rider et al. 2011). Researchers are currently working to extend the range of specific and novel antivirals to other families of pathogens. Since, viruses depend on host cell organism for replication, the selection of target for the designing of effective and safe antiviral drugs without harming the host cell, is an extremely difficult process. Besides this, owing to the evolution, mutations occur in the viral genome, which contribute to the development of resistance to drugs and thus rendered many drugs ineffective (Lee et al. 2019). The peptides can block infection by targeting either virus or its host. The virus specific antiviral peptides are known as virucidal, as they directly target the viral proteins. Most of the antivirals have been reported to inhibit the development of viruses by targeting its specific regions or components. Various steps of viral life cycle have been targeted for the discovery of novel antiviral drugs, such as viral entry, viral synthesis, or assembly. Due to the extracellular site of action and blockage of viral infection, the viral entry inhibition is marked as an attractive strategy (Chew et al. 2017). Protein–protein interactions (PPIs) are the foundation of important cellular processes and are considered as primary targets for the drug discovery over the last decade (Lee et al. 2019; Teissier et al. 2011). The knowledge of crucial interactors involved in PPIs and their mechanism is necessary to pave way for the selection of suitable target for drug discovery. New approaches in therapeutics include the use of small cyclic molecules, proteins/peptides, nucleic

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acids such as small interfering RNA (siRNA) and small hairpin RNA (shRNA) molecules (Teissier et al. 2011). Among these advanced approaches, peptides as therapeutics is a promising field in the drug discovery (Lau and Dunn 2018). Peptides are the biologically active molecules composed of amino acids residues that disrupt the PPIs. They are small (less than 100 amino acids), and they can be easily synthesized. They are also highly specific and effective even in nanomolar range. The main benefit of using peptide as therapeutics is its hydrolysis by peptidases present in the body, which prevents its accumulation in specific organs and minimizes the toxic side effects (Ali et al. 2013). Previously, the pharmaceutical industries have shown poor interest for the expansion of peptide-based therapeutics because of their extremely poor ADME (absorption, distribution, metabolism and elimination) properties. However, the advanced research enables modifications of the peptides such as synthesis of amino acid enantiomers, addition of chemical compounds and their nanoparticle formulation to overcome the pharmacodynamic flaws of peptides (Gentilucci et al. 2010; Zeng et al. 2018). The advantages offered by the modified peptides have sparked the interest amongst the researchers and companies. Now-a-days peptide as therapeutics has come to forte with nearly 20 new peptides added in clinical trials annually. In fact, the global market of peptides as drugs has reached to billion dollars with currently more than 60 peptides approved by US Food and Drug Administration (FDA) and over 400 peptides being under clinical phase trials (Lau and Dunn 2018). Peptide as therapeutics are approved or are being considered for the treatment of diseases such as cancer, diabetes, cardiovascular diseases and even infection caused by few viruses such as HIV, Herpes, Hepatitis and Influenza virus. Thus, over the years peptide-based therapeutics has added a new dimension as the potential antiviral candidate. This review focuses on the types of peptides approaches that can be used for the identification of the lead peptides against the target protein and the selected advanced techniques reported for the validation of the peptide binding affinity to their targets.

Antiviral Peptides as Therapeutics

The peptides possessing potential to inhibit the virus are considered as antiviral peptides (AVPs). Usually, the AVPs exhibit antiviral effects by inhibiting the virus directly, but their inhibition sites and the mechanism of action vary within the viral replication cycle (Rider et al. 2011). The AVPs can be obtained through different approaches: (1) Computational approach (2) Natural sources and (3) Biological source such as High-throughput screening (Fig. 1). There are many online databases available which contain information regarding experimentally tested antiviral peptides such as Antiviral peptide database (AVPdp) (Qureshi et al. 2013) where 2683 entries of peptides including 624 modified AVPs are compiled till December 2019, while many others are unreported. Since the field of peptides as antiviral is not entirely explored, therefore, many research studies are being undertaken to elucidate the role of peptides in blocking viral infections. The first peptide drug approved for clinical indication, Enfuvirtide (Enf), a 36-amino acid residue peptide, against HIV corresponds to the heptad-repeat (HR2) domain of gp41 (HIV envelope protein). Enf prevents the fusion of HR1 domain to HR2 during HIV formation and blocks HIV infection (Teissier et al. 2011). Similarly, Boceprevir and Telaprevir, both synthetic peptides against Hepatitis C virus (HCV), got approval by FDA in 2011. These peptides act on NS3/4, a protease inhibitor, and interfere with viral replication (Divyashree et al. 2020). Other peptide candidates such as Myrcludex for Hepatitis B and D viruses (HBV and HDV) (Bogomolov et al. 2016), Flufirvitide for Influenza virus (Skalickova et al. 2015), Sifuvirtide for HIV-1 (Yu et al. 2018), IM862 and SCV-07 for HCV and Thymosin α-1 for HBV as well as HCV (Jenssen 2009), are under various phase trials of pre-clinical and clinical studies. We have discussed the different approaches used for the identification of AVPs. Moreover, the few selected methodologies used for the validation of identified peptides as potential AVPs have also been described here. The techniques used in identification and validation of peptides are compared in Table 1.

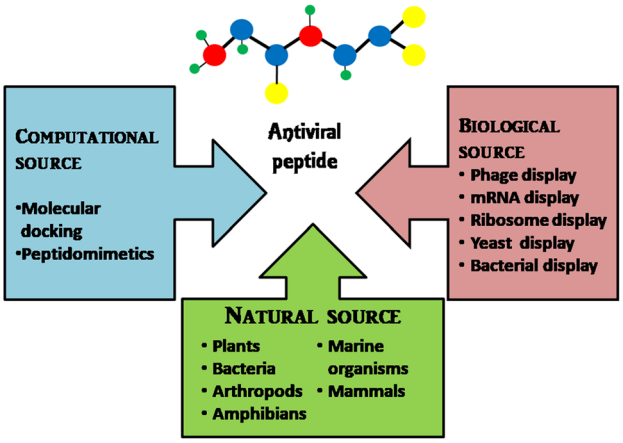

Fig. 1 Different sources of antiviral peptides

Antiviral Peptides Designed from Computational Approach

Computer assisted drug designing process is based on the understanding of structural and functional aspects of the viral machinery. The rational knowledge of the viral proteins and the interactors/cellular partners assists in the selection of target protein. This approach has expedited the process of

Table 1 Comparative analysis of selected techniques applied to identify or validate viral peptides

S. No	Approach	Technique	Characteristics	Advantages	Disadvantages	References
1	Computational	Docking	Virtual screening of target based hits	Expedite the process of drug discovery Size, shape, charge distribution, polarity, hydrogen bonding, and hydrophobic interactions of ligand-receptor complexes can be identified Identification of target sites of the ligand and the receptor molecule Structure–activity based studies	Requires multiple runs to obtain reliable results Limits the flexibility of receptor Less accurate Docking calculations are complex	Nevola and Giralt (2015)
		Peptidomimetics	Mimics of the natural peptides Prevent the protein–protein/protein-peptide interaction by competitive binding	Design the mimic of peptide with enhanced bioavailability Overcome the proteolytic instability of natural peptides Improved receptor selectivity	Offers restricted conformational structures Termini are exchanged with the inversion of their sequence Requires entire understanding of target interaction	Sen et al. (2019)
2	Biological	Phage display	Utilizes phages to display foreign protein/peptide Most adopted system	Rapid identification of target specific phages System suitable for delivering small peptides (< 20 aa) High throughput screening approach Selection of disease specific antigen mimics Selection of organ specific peptides Used in B-cell and T-cell epitope mapping	Library size is limited by phage transformation efficiency ($\sim 10^9$) Allows only natural amino acids Complicated affinity maturation process because of large diversity of proteins/peptides displayed on surface Limits the rapidity of library generation Restricts the size of expressed proteins Limits the intractability of some targets	Matsubara (2012) and Fukunaga and Taki (2012)
		m-RNA display	Uses the transcription and translation machinery extracted from prokaryotic/eukaryotic cells Utilizes covalent mRNA-polypeptide complexes linked through puromycin	Library size as large as ($\sim 10^{12–15}$) Protein expression free from cellular constraints Allows the incorporation of unnatural amino acids Greater diversity as transformation is not required Increased probability of higher affinity hits	Solid surface based biopanning often results in non specific binding Likely to interfere with other molecules due to single stranded form of m-RNA Display larger proteins (> 300 aa) with lower efficiency Not suitable for displaying membrane bound proteins Ribonuclease free environment is required	Wang and Liu (2011)

Table 1 (continued)

S. No	Approach	Technique	Characteristics	Advantages	Disadvantages	References
		Ribosome display	Living cell free technology Utilizes non-covalent ribosome-mRNA-polypeptide complexes for display system	Library size as large as ($\sim 10^{12-15}$) Greater diversity due to not being dependent on transformation efficiency Increased probability of higher affinity hits Suitable for generating toxic, proteolytically sensitive and unstable proteins Random mutations can be introduced Allows the incorporation of unnatural amino acids	Selection stringency is limited System is very sensitive to RNase activity Ribonuclease free environment is required	Dreier and Pluckthun 2011
		Yeast display	Proteins/peptides are usually fused to Aga2 protein subunit of yeast Can be displayed as N- or C-terminal fusion	Displays fully nativemonomeric as well as oligomeric proteins on their surface due to eukaryotic machinery More diverse library as compared to phage system	Library size is smaller ($\sim 10^{6-7}$) than other display systems Allows only natural amino acids Allows the expression of extracellular proteins Complicated affinity maturation process because of large diversity of proteins/peptides displayed on surface	Linciano et al. (2019)
3	Advanced Techniques	Surface Plasmon resonance (SPR)	Label-free binding technique Quantitatively analyzes the real time binding kinetics of two bimolecular interactions	Label-free detection Real-time data monitoring Sensitive and accurate Small sample quantity Ability to Handle Complex Samples Ability to Replicate Measurements	Expensive instrument and sensors Expensive maintenance Low throughput	Patching (2014)
		Biolayer interferometry	Optical analytical technique Quantify real time binding kinetics of bimolecular interactions	Label-free detection Real-time data monitoring No reference channel required Crude sample compatibility System requires less maintenance High throughput Low vibrational/mechanical noise Simple, fast and more accurate results	Requires immobilization of ligand to surface of tip No temperature control Low sensitivity as compared to SPR Poor reproducibility Relatively high sample consumption Results should be cross-validated with SPR	Shah and Duncan (2014)

Table 1 (continued)

S. No	Approach	Technique	Characteristics	Advantages	Disadvantages	References
		Fluorescence Resonance Energy Transfer (FRET)	Radiation less transmission of energy from donor to acceptor Distance-dependant	Simple, sensitive and easily automated Non-radioactive High time resolution High throughput Low reagent consumption	Requires fluorophore labeled molecules Distance of donor–acceptor pair is limited (< 10 nm)	Rogers et al. (2012)

drug designing. Peptides can be identified computationally; via in silico screening using molecular docking. A docking program predicts the target site which is usually known as pocket or protrusion with hydrogen bond donors and acceptors, hydrophobic characteristics and different molecular shapes. Subsequently, library of peptides docked with these pockets results in the highest binding peptide (Nevola and Giralt 2015). For example, peptides have been designed computationally using virtual docking against the surface protein of Zika virus (ZIKV) for its detection. Tetra, penta, hexa and heptapeptide libraries were docked using Open Eye Scientific Software against envelope protein of ZIKV; subsequently, eight peptides were selected. They were further tested by Direct ELISA and out of them, three were delineated with best performance for Zika detection (Mascini et al. 2019). Another method is based on peptidomimetics which mimics the designed targets, prevents the interaction of proteins by competitive binding. Four putative peptide inhibitors were designed against Nipah virus (NiV) proteins using the approach of peptidomimetics and the stability of peptide-protein complexes were analysed using MD simulation (Sen et al. 2019). The in silico methods (Docking and peptidomimetics) can predict the peptide sequence but further validation using in vitro/in vivo approaches is required to establish its biological activity.

Antiviral Peptides Derived from Natural Source

The peptides that exhibit immunomodulatory and inhibitory properties against infection caused by bacteria, fungi, viruses or protozoa, are expressed as naturally occurring antimicrobial peptides (AMPs). The AMPs have been extensively used as substitutes of antibiotics for bacterial infections, but recently their use has been expanded to antiviral therapeutics also (Ahmed et al. 2019a). The naturally occurring AVPs are amphipathic and cationic in nature with net positive charge (Bulet et al. 2004). Moreover, it has been proven that the hydrophobicity of the peptides is an essential property for targeting enveloped viruses (Badani et al. 2014; Wang et al. 2017). These AVPs can be derived from different sources such as plants, bacteria, arthropods, amphibians, marine organisms and mammals with their varied mechanism of action (Zhang and Gallo 2016). A peptide family called cyclotides derived from different plant sources has been proven successfully for their antiviral activity against HIV, Influenza virus and Dengue virus (Ireland et al. 2008; Sencanski et al. 2015; Gao et al. 2010). The small size, cationic and amphipathic nature of the cyclotides facilitates its effective binding to the target and rupture the membrane. This allows the leakage of cell components which further leads to the cell death (Weidmann and Craik 2016). In a study, kalata B1, a cyclotide isolated from the leaves of *Oldenlandia affins* plant, showed destruction of viral particle

at entry step along with inhibition of viral-host membrane fusion, thus exhibiting anti-HIV activity (Henriques et al. 2011). Similarly, a peptide derived from arthropod, *Hyalophora ceecropia,* known as cecropin A showed the inhibitory activity against HIV, Junin virus (JUNV) and HSV by suppression of their gene expression (Wachinger et al. 1998; Albiol Matanic and Castilla 2004). Moreover, in recent studies bovine lactoferrin (bLF) has showed the antiviral activity against three *Aedes* mosquito transmitted viruses: Dengue (DENV), Chikungunya (CHIKV) and Zika virus apart from anti-HBV activity established in a previous study (Li et al. 2009; Carvalho et al. 2017; Chen et al. 2017). The bLF blocks the viral binding to its target site and thus prevents its spread to host cells. Many other AVPs originated from natural source are summarized in Table 2 with their varied mode of action. However, despite promising efficacy, the utility of these peptides is constrained due to weak binding, low stability, other side effects and virus resistance. The shortcomings of AVPs from natural resource need to be addressed so that they can be considered as mainstay antiviral therapeutics.

Antiviral Peptides Identified Through Biological Approach

Methodologies based on in vitro display approach, usually offer genetically encoded peptides with superior quality and high affinity to their targets. Among these methodologies, phage display, mRNA display, ribosome display, yeast display and bacteria display are the most common technologies to generate peptides. The phage display technology is widely used and considered as the most appropriate for the screening of high efficiency peptides. While these technologies have already been thoroughly illustrated in other reviews (Nevola and Giralt 2015), we have focused on the use of selected methodologies for the identification of antiviral peptides and compared them in Table 1.

Phage Display

Phage display technology is an efficient in vitro screening method for the selection of high affinity and target specific peptide binder from a randomly displayed peptide library. The technology involves the fusion of exogenous peptide sequence into the genome of phage, its expression on the surface as fusion product to phage surface protein. The phage displayed libraries thus constructed have $10^{9–10}$ variants at a time. In this method, biopanning is performed in which the target molecule is immobilized on surface and incubated with phage library. The unbound or excess of phage particles are removed by washing and potentially bound phages are eluted by acidic/basic buffer or with appropriate ligand. These recovered phages are amplified by infecting bacterial cells *Escherichia coli* and are used for subsequent rounds of biopanning to obtain target specific phages using affinity selection. The sequencing of DNA isolated from binding phage, validated by ELISA, helps to identify peptide sequence (Fukunaga and Taki 2012; Matsubara 2012). A peptide named P3 against Japanese encephalitis virus (JEV) host fusion has been identified as the potential AVP using phage display library. The screened peptide has shown the highest affinity to domain III of JEV envelope glycoprotein assessed by Biolayer interferometry and IC_{90} of ~ 100 µM and IC_{50} of ~ 1 µM in JEV infected BHK-21 cells (Wei et al. 2019). Similarly, an analogous study conducted by de la Guardia et al. (2017) identified three peptides against the domain III of DENV envelope protein to block the DENV infection. Further these peptides were non-toxic to the target cells. Moreover, the same approach has also been used to identify peptides targeting non-structural viral protein: RNA-dependent RNA polymerase (NS5B) of HCV, by screening a library composed of disulfide-constrained heptapeptides (Amin et al. 2003). In another study, a novel heptapeptide was identified using random peptide phage library which inhibited the integration of HIV genome into the host (Desjobert et al. 2004). The most important advantage of this technology over others is its high rate of mutability with affinity selection, which widely employs the screening of phage displayed peptides for identification of potential AVPs. There are many other AVPs derived from the utilization of phage display technology which are summarised in Table 3.

mRNA Display

mRNA display technology utilizes the covalently bonded mRNA-polypeptide complexes formed during in vitro translation, which are linked through puromycin (an analogue of the 3′tyrosyl-tRNA along with mimics of adenosine and tyrosine) via A- site of ribosome. The complexes with desired functions are allowed to bind to the immobilized target protein, reverse transcribed to cDNA and amplified via Polymerase chain reaction (PCR). This enables the reinforcement of DNA template library for next round of screening (Cotten et al. 2012; Newton et al. 2019). The most successful use of mRNA displayed peptide library was described by Litovchick and Szostal (2008), in which they have screened potential AVPs using cyclic peptide-mRNA fusion library targeting Internal ribosomal entry site (IRES) of HCV for inhibition of virions. Another use of this technology was reported for the reverse engineering of peptide vaccines for HCV. High affinity peptides to neutralizing monoclonal antibodies (mAbs) of HCV were selected in this study and used for peptide-based vaccine development (Guo et al. 2015).

Table 2 Selected AVPs derived from natural sources and their mode of action against virus

S. No	Peptide	Source	Characteristics	Targeted virus	Mode of action/ activity	Reference
I	Plant					
1	2 kDa peptide	Seeds of *Sorghum bicolor*	2 kDa, cationic and amphipathic peptide	HSV-1	Inhibition of cell entry by masking essential viral envelope proteins	Camargo et al. (2008)
2	Cyclotide: vhl-1	Leaves of *Viola hederaceae*	31 amino acid cyclic cystine knot like peptide	HIV	Inhibition of viral fusion by disrupting the lipid envelope	Wang et al. (2008)
3	Cycloviolacin VY1	*Viola yedoensis*	31 amino acid peptide, three conserved disulphide bonds, bridge like 3-D structure	Influenza H1N1	Inhibition of virus at cell entry	Liu et al. (2014)
4	Kalata B1	Leaves of *Oldenlandia affinis*	Cyclic backbone, knot-like rigid structure, three conserved disulphide bonds	HIV	Inhibition of viral-host membrane fusion	Henriques et al. (2011)
5	Kalata B1-inspired peptide	Derivative of Kalata B1 peptide	Amino acid modifications in kalata B1peptide	DENV	Inhibition of viral replication	Gao et al. (2010)
6	Peptides 2 and 4	*Acacia catechu*	15 amino acids, cationic and amphipathic peptide	DENV	Inhibition at early steps of viral entry	Panya et al. (2019)
7	Sesquin	Seeds of *Vigna sesquipedalis*	7 kDa, cationic, defensin like peptide	HIV	Inhibition of viral replication by hindering reverse transcriptase activity	Jack and Tzi (2005)
II	Bacteria					
8	Locillomycin	*Bacillus subtilis*	Cyclic lipononapeptide	PEDV	unknown	Luo et al. (2015)
9	Surfactin	*Bacillus subtilis*	Cyclic lipopeptide, amphiphilic	HIV, HSV-1, HSV-2, VSV, SIV, NDV, PEDV	Disintegration of lipid envelope and, capsid through ion channel formations	Yuan et al. (2019)
III	Arthropod					
10	Alloferon 1 and 2	Hemolymph of blowfly: *Calliphora vicina*	Linear, nonglycosylated oligopeptide of 13 and 12 amino acid residues, respectively	Influenza virus	Inhibition by activation of natural killer cells and release of interferon	Chernysh et al. (2002)
				HSV-1	Inhibition of viral replication	
11	Alloferon 1-derived peptide	Hemolymph of blowfly: *Calliphora vicina*	Modifications in their N-terminal portions of Alloferon1 peptide	CBV-2	Inhibition of viral replication	Kuczer et al. (2010)
12	Bmkn2-7	Venom of scorpion: *Mesobuthus martensii*	13-amino acid residues, basic, alpha-helical peptide	HIV-1	Inhibition by direct interaction with viral particle	Chen et al. (2012)
13	Cecropin A	Moth *Hyalophora cecropia*	37-amino acid linear, cationic peptide	HIV; HSV-1 and 2; JUNV	Suppression of viral gene expression	Wachinger et al. (1998) and Hultmark et al. (2005)

Table 2 (continued)

S. No	Peptide	Source	Characteristics	Targeted virus	Mode of action/ activity	Reference
14	Ctry2459	*Chaerilus tryznai*	13-amino-acid residue, helical and amphipathic peptide	HCV	Inhibition by inactivating the viral particles, virucidal activities and suppressed the established infection at cellular level	Hong et al. (2013)
15	Ctry2459-H2		Histidine-rich Ctry2459 peptide			
16	Ctry2469-H3		Histidine-rich Ctry2459 peptide			
17	Eva1418	Venom of scorpion: *Euscorpiops validus*	Helical and amphipathic peptide	HSV-1	Inhibition by disruption of initial steps of infection	Zeng et al. (2018)
18	Eva1418-FH5		Histidine rich derivative of Eva1418 peptide		Enhanced inhibition activity with lowest cytotoxicity	
19	Hp1090	Venom of Scorpion: *Heterometrus petersii*	Amphipathic α-helical peptide	HCV	Inhibition of viral replication	Yan et al. (2011)
20	Hp1239	Venom of Scorpion: *Heterometrus petersii*	Amphipathic α-helical peptide	HSV-1	Inhibition of cell entry by blocking viral-host membrane fusion	Hong et al. (2014)
21	Hp1036					
22	Lactarcin 1	Venom of spider: *Lachesana tarabaeve*	Amphipathic α-helical peptide	DENV	Inhibition of viral replication by binding to viral protease	Rothan et al. (2014)
23	Mastoparan	Venom of wasp: *Vespula lewisii*	14 amino acid residues, cationic, amphipathic α-helical peptide	VSV, HSV-1, flaviviruses	Inhibition of cell entry by disruption of envelope	Moreno and Giralt (2015)
24	Mastoparan 7	Venom of wasp: *Vespula lewisii*	Derivative of Mastoparan peptide	VSV	Inhibition of cell entry by disruption of envelope	Sample et al. (2013)
25	Melittin	Venom of bee: *Apis mellifera*	26 amino acid linear cytolytic peptide with no disulfide bridge	HIV-1; HSV-1 and 2; JUNV	Inhibition of cell entry by disruption of envelope	Albiol Matanic and Castilla (2004), Galdiero et al. (2013), and Hood et al. (2013)
26	Mucroporin-M1	Scorpion: *Lychas mucronatus*	Cationic host defense peptide	MeV, Influenza-H5N1; SARS-CoV; HIV-1	Virucidal activity	Li et al. (2011)
				HBV	Inhibition of viral replication by decreasing expression of important HBV replication factors	Zhao et al. (2012)
IV	Amphibians					
27	Dermaseptins S3, S4	Frogs of *Phyllomedusa* genus	cationic, amphipathic and α-helical peptide	HSV-1 and 2, HIV	Inhibition at cell entry step by targeting viral envelopes	Lorin et al. (2005)
28	Dermaseptin derived peptide	Frogs of *Phyllomedusa* genus	cationic, amphipathic and α-helical peptide	Rabies virus	Inhibition at cell entry step Virucidal mechanism of action	Bergaoui et al. (2013)
29	HS-1	Skin of Anuran: *Hypsiboas semilineatus*	cationic, amphipathic and α-helical peptide	DENV 2 and 3	Inhibition at cell entry step by targeting viral envelopes	Monteiro et al. (2018)

Table 2 (continued)

S. No	Peptide	Source	Characteristics	Targeted virus	Mode of action/ activity	Reference
30	Magainin I and II	Frog: *Xenopus laevis*	23 amino acid residues, lysine-rich, cationic, amphipathic and α-helical peptide	HSV-1 and 2	Inhibition at cell entry step by disrupting the structure of viral envelope proteins; virucidal activity	AlbiolMatanic and Castilla (2004)
31	Magainin-II derived peptide	Frog: *Xenopus laevis*	alanine-substituted magainin-2 amide along with three other peptides	VV	Virucidal activity by attacking its envelope	
32	Temporin B	Frog: *Rana temporaria*	10–50 amino acids, cationic, amphipathic and α-helical peptide	HSV-1	Inhibition at entry step by interfering in cell-to-cell spread of the virus	
33	Urumin	Indian frog: *Hydrophylax bahuvistara*	27-residues, cationic, amphipathic and α-helical peptide	Influenza-H1N1 and H1N2	Inhibition at cell entry step by targeting cell receptors	Holthausen et al. (2017)
V	Marine organisms					
34	Callipeltin A	*Callipeltin sp.*	Cyclic depsidecapeptide	HIV-1	Inhibition of virion entry	Zampella et al. (1996)
35	Celebeside A-C	*Siliquariaspongia mirabilis*	Cyclic depsipeptides	HIV-1	Inhibition of virion entry	Plaza et al. (2009)
36	Clavanin A	Tunicate: Styelaclava	23 residues alpha-helical peptide with amidated C-terminal	Rotavirus Denovirus	Inhibition by interfering with viral membranes	Carriel-Gomes et al. (2007)
37	Homophymine A	*Homophymia sp.*	Cyclic depsipeptide	HIV-1	Inhibition of virion entry	Zampella et al. (2008)
38	Koshikamides F	*Theonella swinhoei*	17-residue cyclic depsipeptides	HIV-1	Inhibition by blocking HIV entry into T cells	Plaza et al. (2010)
39	Koshikamides H	*Theonella cupola*	17-residue cyclic depsipeptides	HIV-1	Inhibition by blocking HIV entry into T cells	
40	LvHcL48	Hemocyanin of shrimp: *Litopenaeus vannamei*	79 amino acid fragment	WSSV	Inhibition of the transcription and proliferation possibly by binding to the viral envelope protein	Zhan et al. (2018)
41	Microspinosamide	*Sidonops microspinosa*	cyclic depsipeptide incorporating 13 amino acid residues	HIV	Inhibition of cytopathic effects of the infection	Rashid et al. (2001)
42	Mirabamide A, C and D	Siliquaria spongia mirabilis	Cyclic depsipeptides	HIV-1	Inhibition at the early stages of virus entry	Plaza et al. (2007)
43	Mirabamides E, F, G, and H	Sponge: *Stelletta clavosa*	Cyclic depsipeptides	HIV-1	Inhibition at entry step by disruption of viral membrane fusion	Lu et al. (2011)
44	Mollamides B	Tunicate: *Didemmummolle*	Cyclic hexapeptide	HIV	unknown	Donia et al. (2008)
45	Mutremdamide A	*Theonella swinhoei*	Sulfated cyclic depsipeptide	HIV-1	Inhibition by blocking HIV entry into T cells	Plaza et al. (2010
46	Neamphamide A	*Neamphius huxleyi*	Cyclic depsipeptide	HIV-1	Inhibition of virion entry	Oku et al. (2004)

Table 2 (continued)

S. No	Peptide	Source	Characteristics	Targeted virus	Mode of action/ activity	Reference
47	Nkl_{71-100}	Turbot: *Scophthalmus maximus*	five-helix bundled structure stabilized by three intra chain disulphide bonds	SVC	Inhibition by not binding of viral particles to host cells and fusion of virus and cell membranes	Falco et al. (2019)
48	Papuamide A	Tunicate: *Didemmum molle*	Cyclic depsipeptides	HIV	virucidal mechanism	Andjelic et al. (2008)
49	Piscidin 1	Mast cells of hybrid Striped bass (fish)	22 amino acid, α–helical and amphipathic peptide	PRV	Inhibition by direct interaction with virus	Hu et al. (2019)
50	Pa-MAP 1	Polar fish: *Pleunorectus americanus*	an alanine-rich α-helix peptide composed of eleven amino acid residues with three imperfect motif repetitions	HSV-1 and 2	Virucidal mechanism of action, Inhibition at entry step by interacting viral surface glycoprotein	Migliolo et al. (2012)
51	P34	Intestinal contents of *Leporinus* sp. (fish)	Anionic, thermostable, hydrophobic, lipidic peptide	EAV, FHV-1	Virucidal activity	Castro et al. (2014)
				BoHV-1	Inhibition of the viral penetration	Castro et al. (2017)
52	Stellettapeptin A and B	*Stelletta clavosa*	Cyclic and nonribosomal depsipeptides	HIV-1	Inhibition of cytopathic effects of the infection	Shin et al. (2015)
53	TheopapuamideA	*Theonella swinhoei*	Cyclic depsipeptides	HIV-1	Inhibition of virion entry	Andjelic et al. (2008)
54	Theopapuamide B-D	*Siliquariaspongia mirabilis*	undecapeptides with an N-terminal fatty acid moiety	HIV-1	Inhibition by disruption of viral membrane	Plaza et al. (2009)
VI	Mammals					
55	α-Defensin HNPs 1, 2 and 4	Human neutrophil	18 to 45 amino acid residues	HIV-1	Inhibition at cell entry step	Wu et al. (2005)
56	α-Defensin HNPs 1	Human neutrophil	cationic charge, amphipathic properties and predominance of β sheets stabilized by three disulfide bonds	Influenza A	Inhibition of viral replication	Salvatore et al. (2007)
57	β-defensins hDB-2	Epithelial cells	cysteine-rich, cationic peptides	HPV; VZV; HIV	Inhibition at cell entry as well as viral replication by late reverse transcripts and nuclear import	Meyer-Hoffert et al. (2008) and Crack et al. (2012)
58	β-defensins hBD-3	Epithelial cells		HPV; VV; VZV; HIV	Inhibition of viral replication	Quinones-Mateu et al. (2003), Howell et al. (2007), Gwyer Findlay et al. (2013)

Table 2 (continued)

S. No	Peptide	Source	Characteristics	Targeted virus	Mode of action/ activity	Reference
59	Cathelicidin LL-37	Human neutrophil granules	12 to 88 amino acid residues, cationic, α-helical and amphipathic peptide	VZV; VV; HSV-1; HIV; RSV; Influenza A; HCV; DENV; ZIKV; VEEV	Inhibition of cell entry by disruption of envelope	Sørensen et al. (2001), Barlow et al. (2011), Tripathi et al. (2015), Matsumura et al. (2016), Alagarasu et al. (2017)
				Adenovirus; Aichi virus; Rhinovirus	Inhibition of cell entry	Gordon et al. (2005), Sousa et al. (2017), Ahmed et al. (2019b)
60	CYVIP	Human hemofiltrate	71-amino-acid, cationic peptide	HCMV HSV-1	Inhibition of cell entry by interacting the host cell receptors	Borst et al. (2013)
61	Indolicidin	Bovine neutrophils	Tridecapeptide amide	HIV	Inhibition by membrane-disruption	Robinson et al. (1998)
62	Lactoferrin	Mammals' milk	Hydrophobic, cationic, and helical peptide	CMV; HSV-1and 2; Adenovirus; Rotavirus; Poliovirus; RSV; HIV; Influenza; HCV; HBV DENV; CHIKV; ZIKV	Inhibition at cell entry as well as viral replication	Van der Strate et al. (2001), Li et al. (2009), Carvalho et al. (2017), Chen et al. (2017)
63	Lactoferricin	Derivative of lactoferrin	Amphipathic, cationic peptide corresponds to lactoferrin fragment 17–41	CMV, HIV-1, HPV	Inhibition at cell entry step	Andersen et al. (2001), Mistry et al. (2007), Li et al. (2009), Wang et al. (2016)
				HSV-1and 2	Inhibition of viral replication by interfering the host cell microtubules	Marr et al. (2009)
64	Protegrin-1	White blood cells of swine	18 amino acid residues, cyclical, β -sheets and cationic	DENV	Inhibition of viral replication by binding to viral protease	Rothan et al. (2012)

Human immunodeficiency virus: HIV; Dengue virus: DENV; Herpes simplex virus 1 and 2: HSV-1 and HSV-2; Porcine epidemic diarrhea virus: PEDV; Vesicular stomatitis virus: VSV; Simian immunodeficiency virus: SIV; Newcastle disease virus: NDV; Coxsackie virus B2: CBV-2; Junin virus: JUNV; Hepatitis C virus: HCV; Measles morbillivirus: MeV; Severe acute respiratory syndrome coronavirus: SARS-CoV; Hepatitis B virus: HBV; Vaccinia virus: VV; White spot syndrome virus: WSSV; Carp sprivivirus: SVC; Pseudorabies virus: PRV; Equine arteritis virus: EAV; Feline herpes virus type-1: FHV-1; Bovine herpesvirus1: BoHV-1; Human papillomavirus: HPV; Varicella zoster virus: VZV; Respiratory syncytial virus: RSV; Zika virus: ZIKV; Venezuelan equine encephalitis virus: VEEV; Human cytomegalovirus: HCMV; Cytomegalovirus: CMV; Chikungunya virus: CHIKV

Ribosome Display

Ribosome display is an entirely in vitro and cell-free system which makes it efficient in comparison to other display systems (Nevola and Giralt 2015). In this system, the coupling of genotype and phenotype is essential for the selection of high affinity peptides from their pool. During in vitro translation, the association between the mRNA, ribosome and the nascent polypeptide leads to a stabilized protein-ribosome-mRNA complex. This ternary complex is feasible due to the presence of spacer sequence, without stop codon, inserted into the DNA library coding for proteins/peptides. The spacer ensures that the peptide folds properly and stays attached to the mRNA and ribosomes. These specific ribosomal complexes that display folded peptides are then allowed to bind to the immobilized target and the non-specific ones are washed off. The mRNA complexes having bound polypeptide chains are recovered and their sequences are obtained (Zahnd et al. 2007). A large library that contains 10^{13}–10^{14} clones can be screened as it is not dependent on the living cell system and is free of any bias. This technology has numerous advantages above others as the diversity of library depends on the number of available ribosomes and mRNA in the system rather restricted by the bacterial transformation efficiency (Dreier and Pluckthun 2011). Moreover, such system allows insertion of random mutations at any round of selection since library has not been transformed after any diversification step. The ribosomal display technology has opened a new insight for using peptide inhibitors for early diagnostic as well as therapeutic agent. For instance, the peptide inhibitor against envelope protein E2 of HCV was identified using ribosomal display library. After extensive selection of 13 rounds, 12-mer peptides were generated. This peptide named PE2D has not only being verified to bind E2 protein but also blocks the virus entry inside hepatocyte cells (Chen et al. 2010).

Yeast Display

The main advantage of the yeast display system over the others is the complete exposure of the peptides/protein for fusion and its compatibility with the fluorescence-activated cell sorting (FACS), which enables the high-throughput screening and characterization of protein/peptide combinatorial libraries (Linciano et al. 2019). Moreover, it also allows the expression of proteins with post translational modifications which has encountered the problem of misfolding in the field of antibody engineering (Mei et al. 2017). *Saccharomyces cerevisiae* strain based on the Aga1–Aga2 proteins is the most widely used display system. In this system, the protein/peptide is displayed either as N- or C-terminal fusion to the Aga2 protein of yeast cell, which is linked to the Aga1 via disulphide bonds. Every yeast cell exhibits ~ 10^4–10^5 copies of the Aga2 fusion protein/peptide on its surface though the expression of individuals may vary. The construct of yeast cell also contains two epitope tags at the N and C terminus of Aga2 fusion protein, which facilitates the real time quantification of their expression using flow cytometry. Moreover, the tags also enable to estimate and quantify the binding of the target via different labelling approaches (Linciano et al. 2019). Though yeast display system is a valuable platform for screening purpose, however, the library size is restricted due to limited transformation efficiency of yeast. Another major limitation of yeast display is complicated affinity maturation process in comparison to other systems. Besides, these drawbacks, this technology has provided a wide application of high throughput screening in peptide engineering and a platform to study protein–protein/peptide interactions in vivo. In a recent study, this technology was used to screen the hits from a grafted C-peptide library of HIV gp41 against N-peptide trimer of HIVgp41. As a result, four hits suppressed the HIV entry better than others (Tennyson et al. 2018).

Application of Advanced Techniques to Validate Identified Peptides

Another challenging task in the intervention of antiviral peptides is the corroboration of the binding of selected peptides to the target protein, and their antiviral efficacy. Various techniques have been developed to evaluate the PPIs in vitro/in vivo such as Surface Plasmon Resonance (SPR), Optical based Biolayer interferometry, Fluorescence Resonance Energy Transfer (FRET), Nuclear Magnetic Resonance (NMR), Isothermal Calorimetry (ITC), yeast two hybrid display, microscopic visualization and many more. Some of them can be used for the binding evaluation of the peptides to their target proteins. Since various reviews and reports are available on PPIs detection methods in detail (Nevola and Giralt 2015), this review focuses only on the recent techniques used to determine/ validate the peptide binding efficiency (Table 1).

Surface Plasmon Resonance (SPR)

SPR is an optical based detection and label-free technique which utilizes the protein in small amount for the real time quantification and evaluation of the binding affinity as well as kinetics between peptide and target protein (Patching 2014). The binding affinity between interacting partners is measured via small variation in the refractive index at sensor surface. This response change is calculated as the change in the angle of resonance of refracted light when flowing analyte binds to the immobilized ligand. The change in the angle of resonance is measured in the form of resonance unit

Table 3 Characteristics of the AVPs derived from Phage display technology

S. No	Peptide sequence	Library used	Targeted virus	Targeted protein	References
1	GSHHRHVHSPFV	12-mer peptide library: New England Biolabs (NEB)	Avian infectious bronchitis virus	Purified whole virus	Peng et al. (2006)
2	HAWDPIPARDPF	12-mer peptide library (NEB)	Avian influenza A virus-subtype H5N1	H5N1 viruses	Wu et al. (2011)
3	AAWHLIVALAPN				
4	ATSHLHVRLPSK				
5	NDFRSKT	7-mer disulfide constrained peptide library (NEB)	Avian influenza virus H9N2	AIV sub-type H9N2 virus particles	Rajik et al. (2009)
6	HSIRYDF	7-mer peptide Library (NEB)	Bovine ephemeral fever virus	Neutralization site 1 of glycoprotein: G1	Hou et al. (2018)
7	YSLRSDY				
8	DRATSSNA	Octapeptides peptide library	Classical swine fever virus	Envelope protein: E2	Yin et al. (2014)
9	SYQSHYY	7-mer peptide Library (NEB)	Dengue virus	Recombinant dengue envelope protein and its domain III	de la Guardia et al. (2017)
10	STSFWIT				
11	ELLASPW				
12	CWSFFSNIC	7-mer disulfide constrained peptide library (NEB)	Hepatitis B virus	Full-length HBcAg	Ho et al. (2003)
13	KHMHWHPPALNT	12-mer peptide library (NEB)	Hepatitis B virus	PreS1 region of L-protein	Wang et al. (2011)
14	WTDMFTAWWSTP	M13-based 12-mer peptide library	Hepatitis B virus	Thio-PreS	Deng et al. (2007)
15	FPWGNTW	7-mer disulfide constrained peptide library (NEB)	Hepatitis C virus	NS5B (del 21-His) protein	Amin et al. (2003)
16	ATWVCGPCT	Phage-displayed nonapeptide library (PVIII9aa)	Hepatitis C virus	mAb JS-81 against CD81	Cao et al. (2007)
17	WPWHNHR	heptapeptide M13 phage-display library	Hepatitis C virus	Truncated envelope protein E2	Lu et al. (2014)
18	RINNIPWSEAMM	libraries of random 12-mers, 7-mers, and cyclic 9-mers	Human immunodeficiency virus	Envelope glycoprotein gp120	Ferrer and Harrison (1999)
19	VSWPELYKWTWS	7-mer disulfide constrained peptide library; 12-mer peptide library (NEB)	Human immunodeficiency virus	mAb VRC01	Chikaev et al. (2015)
20	FHNHGKQ	7-mer peptide library (NEB)	Human immunodeficiency virus	HIV-1 Integrase	Desjobert et al. (2004)
21	GWWYKGRARPVSAVA	Pentadecapeptides peptide library	Influenza virus A	Monolayer of the ganglioside:GM3	Matsubara et al. (2009)
22	RAVWRHSVATPSHSV				
23	SENRKVPFYSHS	12-mer peptide library (NEB)	Japanese encephalitis virus	Domain III of the virus envelope glycoprotein	Zu et al. (2014)
24	TPDCTRWWCPLT	12-mer peptide library (NEB	Japanese encephalitis virus	E protein	Wei et al. (2019)
25	RLNNRARIILRA	12-mer peptide library (NEB)	Mink enteritis virus	Purified whole virus	Zhang et al. (2012)
26	LAHKSRLYERHM				
27	CTLTTKLYC	7-mer disulfide constrained peptide library (NEB)	Newcastle disease virus	Inactivated whole virus	Ramanujam et al. (2002)
28	EVSHPKVG	Heptapeptide library-pSKAN8-HyA library	Newcastle disease virus	Inactivated whole virus	Ozawa et al. (2005)
29	SGGSNRSP				
30	WVTTSNQW				

Table 3 (continued)

S. No	Peptide sequence	Library used	Targeted virus	Targeted protein	References
31	IQTAFNQGA	7-mer disulfide constrained peptide library (NEB)	Porcine reproductive and respiratory syndrome virus	mAb N3H2 against nucleocapsid protein	Liu et al. (2012)
32	HRILMRIR	12-mer peptide library (NEB)	Porcine reproductive and respiratory syndrome virus	ORF1b	An et al. (2005)
33	CHWMFSPWC	Random heptapeptide library flanked by cysteines	Puumala orthohantavirus	Inactivated whole virus	Heiskanen et al. (1997)
34	TATTEK	12-mer peptide library (NEB)	West Nile virus	Non-structural protein 1	Sun et al. (2011)
35	VVDGPETKEC				
36	P9 peptide	Peptide library (Spring Bioscience)	West Nile virus	Recombinant E protein	Bai et al. (2007)

(RU), where 1RU is equivalent to the 10^{-4} deg/10^{-12} gmm^{-2} angle shift. It has become the gold standard in research, typically characterizes the interaction between two molecules in which one is in mobile state and the other is fixed on a gold film. This technique can be used to screen the library of molecules for their binding affinity against a single soluble protein which is immobilized on the sensor surface (Tang et al. 2010). Thus, SPR has emerged as a powerful technique in therapeutic intervention. It can also be adapted to study the interactions involving complicated proteins in situ, such as, membrane-bound proteins, ion channels and other growth, immune and cellular receptors, which are considered as potential targets for drug discovery (Patching 2014). Bai et al. (2007) have investigated the affinity interaction of screened peptides to the Envelope protein of West Nile virus (WNV) using SPR, in which they found peptide P9 to have the highest affinity to the target. Besides this, in another study, the binding of Helix-A peptide to the neuronal microtubules (MTs): β-tubulin was determined by SPR. Helix-A peptide prevents the binding of the gp120 protein of HIV to the β-tubulin, a neuronal MT and possesses neuroprotective activity (Avdoshina et al. 2019). Moreover, the SPR has been used as a ligand screening strategy for Influenza virus and HSV-1, in which the technique enables the continuous screening of inhibitors that inhibit the viral entry. The major advantage of this technique is the use of minimal amount of immobilized viral surface proteins or receptors as compared to other techniques (Kumar 2017).

Biolayer Interferometry

Another optical based and label-free technique is the Biolayer interferometry (BLI) which validates the interaction between two molecules by quantifying the change in an interference pattern. The target molecule is immobilized on the tip of fiber optic biosensor that moves toward the wells containing the binding partner present in solution. The association and dissociation of the binding partner with the immobilized molecule is monitored by BLI, leading to the generation of optical thickness at the tip of biosensor that produces an optical interference pattern. This pattern can be quantified and used to determine real time kinetic rates of binding and dissociation (Shah and Duncan 2014). Thus, it has become a valuable tool for monitoring interactions between small molecules in the field of drug discovery. This technique is advantageous over others as nonspecific and non ideal interactions can be differentiated in initial steps by examining their binding response and moreover, it has low false positive rate. Besides, the varied flow rate, available unbound molecules and the refractive index of adjacent medium do not affect the obtained interference pattern, which is the unique property of this technique (Wartchow et al. 2011). In this context Zu et al. (2014) have analysed the real time binding affinity of chemically synthesized screened peptides to the Domain III of JEV envelope protein and reported peptide P3 possessed the highest affinity.

Fluorescence Resonance Energy Transfer (FRET)

FRET is a sensitive method to investigate the interaction of proteins with large diverse set of peptides/proteins libraries for high throughput screening efficiently. This technique is reliable on the distance-dependent transfer of energy between dye-labelled molecules, where the excited donor fluorophore transfers its energy to an acceptor chromophore (Rogers et al. 2012). This energy transfer determines the ratio metric signal generated by the reduction in fluorescence of donor molecule and the increment in fluorescence of acceptor molecule. The technique of FRET can be used as both screening as well as validation method. In view of this, various FRET based studies have been reported for the identification of potent inhibitors against several viral proteases such as SARS coronavirus 3CLpro protease, DENV NS2B-NS3 protease, WNV Serine Protease, HCV NS3/4A protease

and HIV protease. Similarly, a FRET based proteolytic assay was used to screen the compounds against CHIKV capsid protein (Aggarwal et al. 2015).

Challenges to the Peptide as Therapeutic Use

Several limitations that obstruct the way of peptide to be a successful therapeutic drug, are its instability, short half-life, lower potency, inability to cross membrane barriers and poor bioavailability due to protease degradation (Ali et al. 2013). The main challenge is to overcome these limitations and to achieve the desired efficacy for the required time span. Various modifications have been employed to enhance the stability and physiochemical properties of the peptides (Gentilucci et al. 2010). For instance, the conjugation of peptide to polymers such as polyethylene glycol (PEG) has enhanced the stability of peptides by increasing their molecular weight (Chew et al. 2017). Likewise, the bioavailability was improved by balancing the aqueous solubility via replacement of redundant hydrophobic amino acids to charged/polar residues (Mant et al. 2009; Wu et al. 2010). Moreover, there are two computational softwares based on support-vector machine (SVM) to predict the solubility of the peptides, thus assisting in the designing and optimising the peptide bioavailability (Lee et al. 2019). In addition to the strategies involved in the improvement of peptide properties, the delivery of peptides has also been improved by linking of peptides to the cell penetrating peptides (CPPs) to enhance their cell permeability (Chew et al. 2017). CPPs are general peptides (< 30 amino acids) derived from natural/unnatural sources or chimeric sequences, considered as promising carrier for successful delivery of therapeutic molecules varying from small chemical molecules, liposomes, proteins, peptides and nucleic acids for in vitro as well as in vivo applications (Heitz et al. 2009). Alternatively, peptides can be encapsulated in nanoparticles for efficient delivery, or administered through primary parental or transdermal routes with variations such as prefilled syringes, auto injectors and biodegradable micro needles (Lee et al. 2019). These modifications help to address the challenges of poor ADME properties of non-modified peptides (Lau and Dunn 2018).

Conclusion

In summary, the discovery of the peptide-based therapies have made a significant impact in the research. Many peptide therapies are available in the clinical and pre-clinical trials which are expected to yield positive results. The various approaches including computational, natural and biological sources provide a wide repository for identification of peptides involved in viral therapeutics. These promising therapeutics/inhibitors are advantageous because of their high specificity, selectivity against target and can be easily developed without the prior structural knowledge of target (except docking). Novel technologies like SPR, BLI and FRET validate the binding of identified peptides to their target which further aid in the selection of high affinity potential AVPs. However, there is a dearth of knowledge in the field of identification and characterization of antiviral therapeutics; further advancement and commercialization of peptide-based therapeutics is warranted.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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