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Review Article

Recent advances in the molecular design and applications of proteolysis targeting chimera-based multi-specific antiviral modality

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ABSTRACT

Viral infections represent a major threat to human health and the global economy; however, most of the currently available antiviral drugs are not fully effective in restricting viral replication and selecting for drug-resistant variants. Targeted protein degradation technologies are promising strategies to avoid or delay the emergence of drug resistance. Among the protein degradation-based multi-specific approaches, proteolysis targeting chimera (PROTAC) is the main strategy applied in the antiviral field. In this review we will introduce the elements and mechanisms of action used by PROTAC technology, as well as the advantages of PROTACs over available antiviral drugs. We also summarize the latest progress in the application of PROTACs in antiviral research, discuss existing challenges and look into future opportunities for antiviral drug discovery.

Keywords: viruses, antiviral drugs, targeted protein degradation, PROTAC, multi-specific drugs

1. INTRODUCTION

Throughout human history viral pandemics have claimed tens of millions of lives. One of the first outbreaks in modern times was the Spanish influenza pandemic in 1918, which infected one-third of the global population and caused up to 100 million deaths worldwide [1]. The human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) and has been responsible for another terrible pandemic, currently infecting 39 million people worldwide. Since the first reported cases of AIDS in 1981 [2], HIV infections have killed approximately 40.1 million people [3]. The new coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is also considered one of the three deadliest viral pandemics in the last 100 years [4], with greater than 767 million confirmed cases and greater than 6.9 million deaths as of 12 July 2023 [5]. These epidemics are examples that illustrate the major impact of viral infections in human health and public safety at a large scale. Medicines are important weapons in the fight against viral infections, and 103 approved antiviral small molecules targeting 10 virus families have been approved to date [6]; however, emergence of drug resistance is still an important matter of concern and therefore it is necessary to explore alternative antiviral strategies to combat viral infections [7-10].

At present there are many small-molecule inhibitors (SMIs) that target protein functions; however, the efficacy of SMIs is limited by the potential emergence of drug resistance. Drug selection pressure facilitates the selection of resistance-associated mutations that constitute a major threat to therapy success [11, 12].

Specific ligands designed to target viral proteins are not very effective in the treatment of diseases and thus need to be combined with other drugs to

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avoid resistance [13]. Of concern, creating compounds with the capacity to target and degrade viral proteins could lead to the loss of function, while showing higher resilience to the emergence of drug resistance [14-16].

Targeted protein degradation (TPD) is a new field of drug development and has an enormous potential for the treatment of severe diseases [17]. This technology takes advantage of the eukaryotic cell ubiquitin-proteasome system and lysosomal degradation pathways to degrade key proteins required for viral replication. PROTAC [18], hydrophobic tags (HyT) [19] and molecular glues [20] are examples of compounds driving the targeted protein to the ubiquitin-proteasome system, while autophagy-targeting chimera (AUTOTAC) [21], autophagosome-tethering compounds (ATTEC) [22], and lysosome-targeting chimaera (LYTAC) [23] help degrade the target protein via the lysosomal pathway.

PROTACs are hybrid molecules containing an E3 ubiquitin ligase-recruiting moiety attached to a ligand that binds to a protein of interest (POI) [24]. Research on PROTAC molecules has mostly concentrated on cancer therapy [25-29], but also in inflammatory, bone-related and neurodegenerative diseases, as well as other pathologies [30-32]. Target specificity and resistance are important requirements for the development of both anti-neoplastic and antiviral drugs. In addition, PROTACs have become the main TPD strategy that has been applied in antiviral research [33]. Therefore, this review provides a concise overview of new strategies for developing antiviral drugs promoting protein degradation through the ubiquitin-proteasome pathway.

2. MECHANISM OF ACTION AND PROTAC DESIGN

2.1 Mechanism of action of PROTACs

PROTACs contain three elements: an E3 ubiquitin ligase ligand; a ligand for the POI; and a linker. The E3 ubiquitin ligase ligand is used to specifically recruit the enzyme complex, while another ligand targets the POI. Both ligands are connected by a linker required to form a stable ternary complex (**Figure 1**) [18, 34]. The target protein is covalently "tagged" with ubiquitin as a consequence of the PROTAC molecule capacity to draw E3 ubiquitin ligases to the POI proximity. The proteasome in the cell is then fed with the protein that has been covalently tagged with ubiquitin for degradation [35]. The ubiquitin-proteasome system acts by covalently tagging the proteins to be degraded with ubiquitin. This tagging process is catalyzed by three enzymes (the ubiquitin-activating enzyme [E1], the ubiquitin-conjugating enzyme [E2], and a ubiquitin-ligase [E3]) [25]. Free ubiquitin is activated by binding to an active cysteine residue (Cys) in E1 through the formation of a thioester bond in an ATP-dependent reaction. Ubiquitin-tagged E1 transfers the ubiquitin moiety to the Cys residue of E2 through a trans-thioesterification reaction, then E3 recruits ubiquitin-tagged E2 and E3 substrates to label the POI with ubiquitin at a Lys residue, which is then recognized and degraded by the proteasome (**Figure 1**) [36-38].

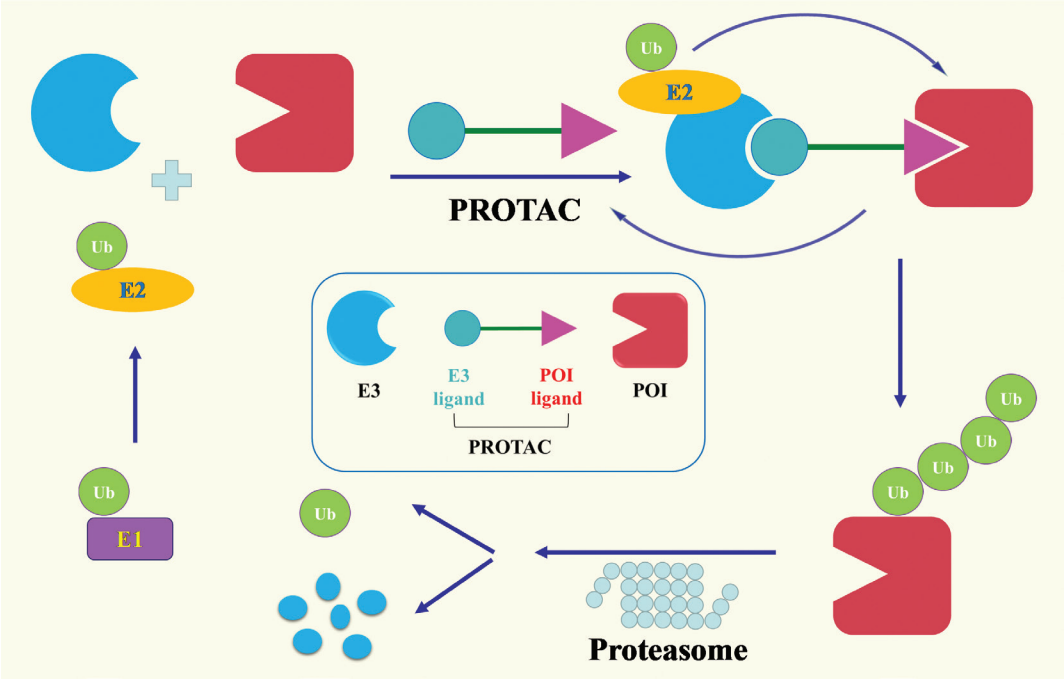

Figure 1 | A mechanistic overview of PROTAC-mediated protein degradation. Ub, ubiquitin (ubiquitylation of the indicated protein); POI, protein of interest.

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2.2 Target-binding molecules

Viruses contain numerous proteins that can be targeted using specific ligands. Indeed, ligand specificity might be used to design PROTACs; however, the ubiquitin and proteasome levels in the host cell are critical for the PROTAC to exert its degradative effects because the complete ubiquitin-proteasome system is the basis of its function. Various studies have shown that binding sites in solvent-exposed regions should be selected based on high-resolution structures, and the occupancy should avoid alterations in binding affinity of the small molecule to the target protein [39]. Therefore, crystal structures of small molecules bound to the POI provide a theoretical basis for the selection of suitable ligand attachment sites. Usually, key interactions are provided by specific atoms of the ligand, such as carboxyl and amino groups, and linkers can be synthesized by different types of reactions (e.g., amide condensation) while maintaining the functionally-relevant groups for binding [40].

The ubiquitin-proteasome system is present within cells. Some proteins that function on the host cell membrane, such as the CD4 receptor and co-receptors (CCR5 and CXCR4) involved in HIV binding [41], may not be suitable for PROTAC strategies. Therefore, when designing PROTACs targeting viral proteins, the functional sites and mechanisms of action should be analyzed first.

2.3 E3 ligands

It is widely known that the size of the ligands and the E3 ligase binding behavior have a major influence on PROTAC activity *ex vivo*. There are 600-700 E3 ligase genes in humans, but fewer than 10 E3 ligases have been used for targeted protein degradation, which is a major limitation for further development of the technique [42]. There are many types of E3 ubiquitin ligases, such as cereblon (CRBN), von Hippel-Lindau (VHL), the tripartite motif-containing protein 25 (TRIM25), and murine double minute 2 (MDM2) [42-45]. Small molecules, which bind to E3 ligase components, are used in antiviral drug development for targeting E3 substrate receptors [45, 46].

2.4 Linkers

The overall degradation efficiency of PROTACs is influenced by the adaptability of the linkers, and by the properties of E3 ligase ligands and the POI. It has been demonstrated that the physicochemical characteristics of PROTACs and the resulting degrading activity depend greatly on the length and composition of the linker [47-49]. Guidelines for the *de novo* design of PROTAC linkers guaranteeing a potent effect do not exist. The linker composition needs to be optimized for each pair of binders, especially in terms of the length and conjugating sites on each ligand. The most potent PROTAC linkers are selected after systematic and extensive iterations, including different chemical motifs.

Alkyl chains, as well as polyethylene glycol and other glycol chains, have all been widely used as PROTAC linkers. Alkyl chains can be easily synthesized and adapted to a variety of chemical applications. Topologic polarity and lipophilicity can be changed to optimize the solubility and cell permeability of PROTACs by adjusting the polyethylene glycol and alkyl moieties. In recent research, rigid links, including heterocyclic scaffolds and aromatic rings, which impose conformational limits to enhance the physicochemical characteristics of degraders, have gradually replaced linear alkyls and ether linkages. In addition, triazole moieties have been frequently used as PROTAC linkers due to their chemical stability and synthesis simplicity through click chemistry [50]. Differences in the linker composition of PROTACs can have a considerable impact in pharmacokinetic characteristics, such as

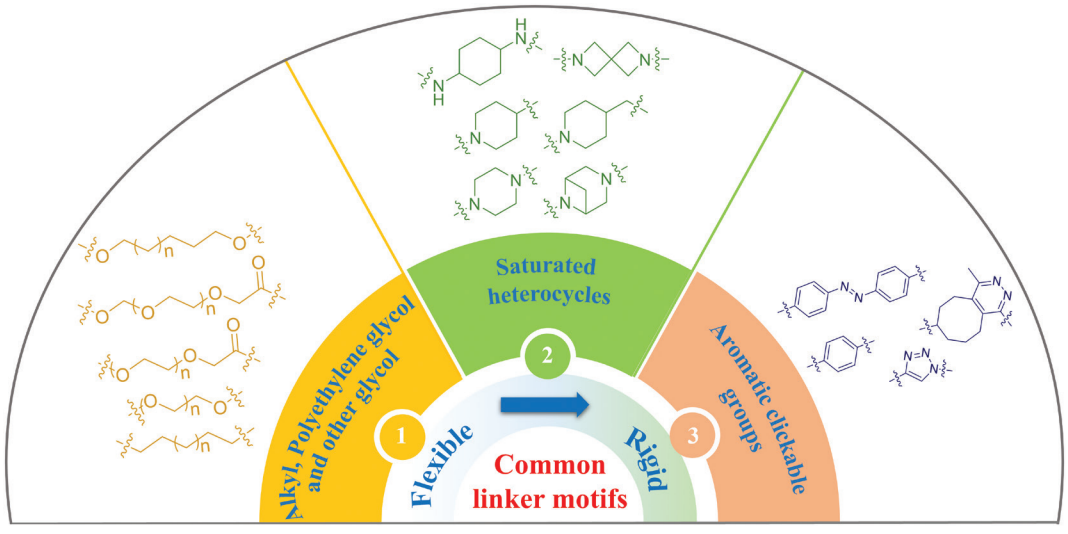

Figure 2 | Common linker motifs used in PROTAC design.

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cellular permeability, metabolic stability, and solubility (**Figure 2**) [44, 49-51].

2.5 Advantages of PROTAC

2.5.1 High catalytic activity. The degradation of POI using PROTAC molecules is a catalytic process in which PROTACs can be dissociated once the target protein has been degraded and remain active and ready to target another protein. This catalytic property makes PROTACs highly effective at low doses [52]. SMIs, which usually act at drug binding sites, are likely to increase the risk of off-target toxicity because significant exposure is often needed to establish sufficient site occupancy to affect downstream biological processes [53].

2.5.2 Avoiding drug resistance. Traditional SMIs use an "occupancy-driven" mode of action to inhibit the function of target proteins to treat disease. This mechanism of action requires high concentrations of inhibitors to occupy the active site of the target, and therefore block the transduction of downstream signaling pathways, such as drug resistance, which often occurs during the clinical treatment of cancer [53, 54].

PROTACs act through an "event-driven" mode of action. Instead of affecting protein function, PROTACs mediate degradation of the disease target protein, which can overcome the drug resistance mechanism of parent SMIs and the drug resistance of existing small molecule drugs [14-16, 55, 56].

2.5.3 High selectivity. It has been reported that the degradation selectivity of PROTACs is not only equal to the target protein ligand but may exceed binding selectivity [57, 58]. The high selectivity of PROTACs may be attributed to degradation, which depends on the formation of the target protein-PROTACs-E3 ligase ternary complex, not just the binding of PROTACs to the target protein. In addition, because the ubiquitination site on the surface of the protein is dependent on the uneven distribution of amino acids provides three-dimensional selectivity, not all proteins targeted by PROTACs can be efficiently ubiquitinated [42, 59].

2.5.4 Degrading the "undruggable" protein targets. SMIs cannot target all proteins. Some proteins are enzymatically inactive, such as transcription factors, scaffolding proteins, and proteins that function through albumin-protein interactions, while other proteins have strong binding forces to endogenous ligands, making it difficult for SMIs to block the binding. These proteins, which account for approximately 80% of the human proteome, are usually referred to as "undruggable proteins" [60].

In contrast to SMIs, PROTACs act in an "event-driven" mode through transient binding to target proteins, complete ubiquitination labeling, and subsequent target protein degradation. Therefore, in addition to the active site, PROTACs can also act on any site on the surface of the protein, which is expected to target "undruggable proteins," broadening the range of target proteins. At present, "undruggable proteins," have been confirmed to be degraded by PROTACs, including transcription factor STAT3 and epigenetic-related protein PCAF/GCN5 [31, 61, 62].

3. NOVEL ANTIVIRAL AGENTS FOR TARGETED PROTEIN DEGRADATION

3.1 New PROTACs in targeted HCV therapy

Hepatitis C virus (HCV) infection is a major cause of liver disease (e.g., chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma), and millions of people are persistently infected worldwide [63]. The HCV NS3 protein has protease and helicase activities that are essential for viral infection; however, only NS3/4A proteases have been used as drug targets [64]. PROTACs tested against HCV have used the viral NS3/4A protease as the target protein with telaprevir (**1**, **Figure 3**), a reversible covalent inhibitor, as a protease-binding ligand. The pyrazine ring of telaprevir was linked to several $CRL4^{CRBN}$ (a well-characterized ubiquitin E3 ligase complex) ligands [65, 66], such as lenalidomide, pomalidomide, and a novel tricyclic imide moiety. Several PROTACs were obtained, and among them DGY-08-097 (**2**, **Figure 3**) was characterized as the most efficient. As observed with telaprevir, all PROTAC molecules inhibit HCV NS3/4A protease activity with IC_{50} (one-half inhibitory concentration) values in the nanomolar range [14].

The induction of NS3 protein degradation by PROTAC molecules containing telaprevir was estimated using an inducible cell line expressing the full-length HCV NS3 protein fused to eGFP linked to mCherry through the foot-and-mouth disease virus (FMDV) 2A ribosomal skipping sequence. The FMDV 2A sequence leads to translation of NS3-eGFP and mCherry, respectively. Therefore, the NS3 protease-eGFP:mCherry fluorescence ratio provides an estimate of the relative amount of protease present in the cells.

Researchers demonstrated that PROTACs reduce the intracellular NS3 protein in a concentration-dependent manner. In addition, it was observed that when the degradation activity was maximal, increasing the concentration of PROTAC molecules led to a rapid decrease in NS3 protein degradation activity. This finding was attributed to the independent engagement of NS3 and CRBN by the degrader, which interferes with the formation of the productive trimeric complex necessary for efficient ubiquitin transfer. This phenomenon is known as a "hook effect," and is similar to that observed in immunoassays when antigen excess limits the effectiveness of antibodies to form immune complexes.

Methylation of the glutarimide moiety prevents binding to the CRBN analogue. Therefore, researchers treated the cells with DGY-08-097 and methylated DGY-08-097 to determine the selectivity of the degraders

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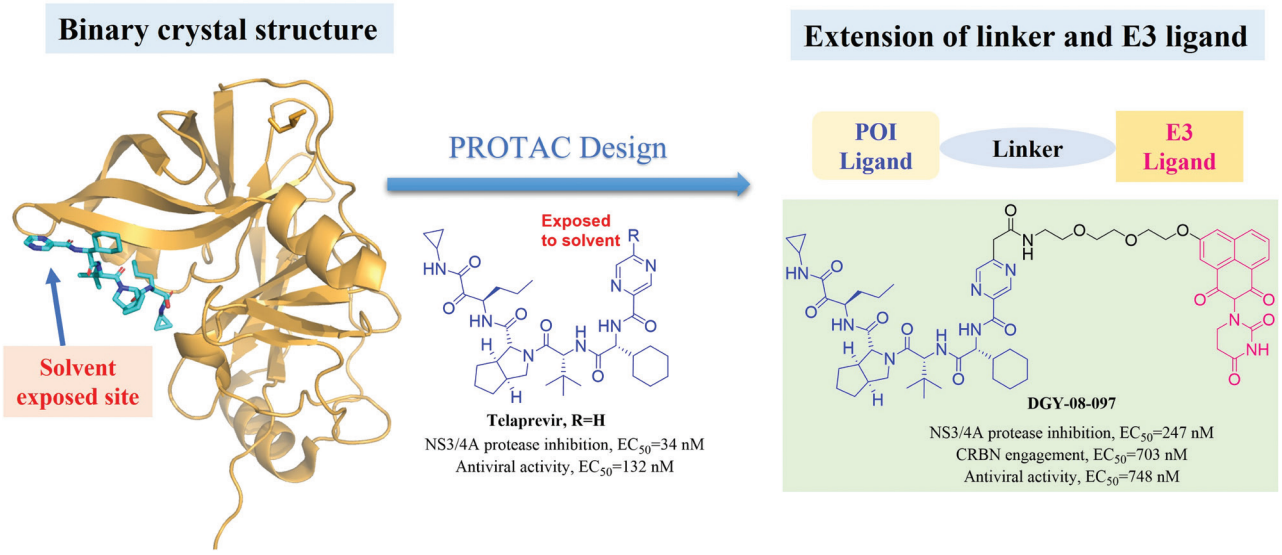

Figure 3 | Crystal structure of telaprevir bound to the HCV NS3/4A protease complex (PDB code: 3SV6), the figure was generated in PyMOL (www.pymol.org), and extension of linker and E3 ligand-based PROTAC technology.

through quantitative mass spectrometry-based proteomics. The HCV NS3 protease was rapidly degraded in cells in the presence of DGY-08-097, but the levels remained unaffected in negative controls exposed to methylated DGY-08-097. These data showed the specificity of PROTACs in degrading the viral protein.

Further experiments confirmed that the degradation of NS3 is indeed achieved by E3 ligase bound to CRBN through the ubiquitin-proteasome system, and that DGY-08-097 is effective in inhibiting HCV replication in a cellular infection model with a median effect concentration (EC_{50}) of 748 nmol/L, which demonstrates that protein degradation contributes to the antiviral activity.

The CRBN-dependent degrader functions of DGY-08-097 and related PROTACs have also been demonstrated using CRBN knockout Huh7.5 cell lines, which are generated with CRISPR editing technology. By comparing the effects of PROTAC molecules on NS3 protein abundance in wild-type and CRBN knockout cells, it has been found that HCV NS3 protein degradation is significantly inhibited in cells lacking CRBN and that telaprevir is not dependent on CRBN for antiviral activity [24].

Finally, telaprevir-based degraders have been shown to be effective against HCV clones carrying resistance-associated mutations (V55A or A156S). Thus, even though the antiviral potency of the PROTAC is significantly lower than telaprevir, new PROTACs are superior in mediating antiviral activity against drug-resistant viruses.

3.2 New PROTACs for anti-influenza virus therapy

3.2.1 Pentacyclic triterpenoid PROTACs, as a class of effective hemagglutinin (HA) protein degraders. A novel class of pentacyclic triterpenoid PROTACs was recently designed by Zhou's lab [67] to facilitate the degradation of influenza virus HA proteins. In a previous study they [68] reported that an oleanolic acid (**3**, OA, Figure 4) derivative with galactose at position 17-COOH (**4**, Y3, Figure 4) exhibited significant anti-influenza A/WSN/33 virus activity with an EC_{50} value at the micromolar level. OA alone has no antiviral activity at high concentrations (EC_{50} > 200 μmol/L), but has a modest capacity to bind HA (K_D = 34 μmol/L; the docking results are shown in Figure 4). OA derivatives are considered good candidates for PROTAC development based on the HA-binding capacity. Two series of compounds were designed and obtained [C1–C3 and V1–V6] (**5**, **6**, Figure 4). These PROTACs contain CRBN and VHL ligands and OA derivatives targeted to the POI (i.e., influenza virus HA in this case).

PROTAC treatment leads to the degradation of the viral HA in 293T cells transfected with an HA-encoding plasmid. While the C1–C3 derivatives containing the CRBN ligand display the typical "hook effect". The PROTACs V1–V3 with the VHL ligand demonstrate degrading capacity at high concentrations. V3 induced ubiquitination and proteasomal degradation of HA in 293T cells infected with influenza virus. In addition, this PROTAC protected mice against influenza A virus-induced weight loss and death.

Surface plasmon resonance (SPR) analysis has shown that OA binds specifically to the viral protein, HA, while the CRBN and VHL ligands are specific for the E3 ligases of CRBN and VHL, respectively. PROTAC molecules, however, show significant affinity for both HA and the corresponding E3 ligase.

Proteasome-mediated degradation is critical for PROTAC efficiency. MG-132, a proteasome inhibitor, completely blocks the effects of PROTAC [69]. As

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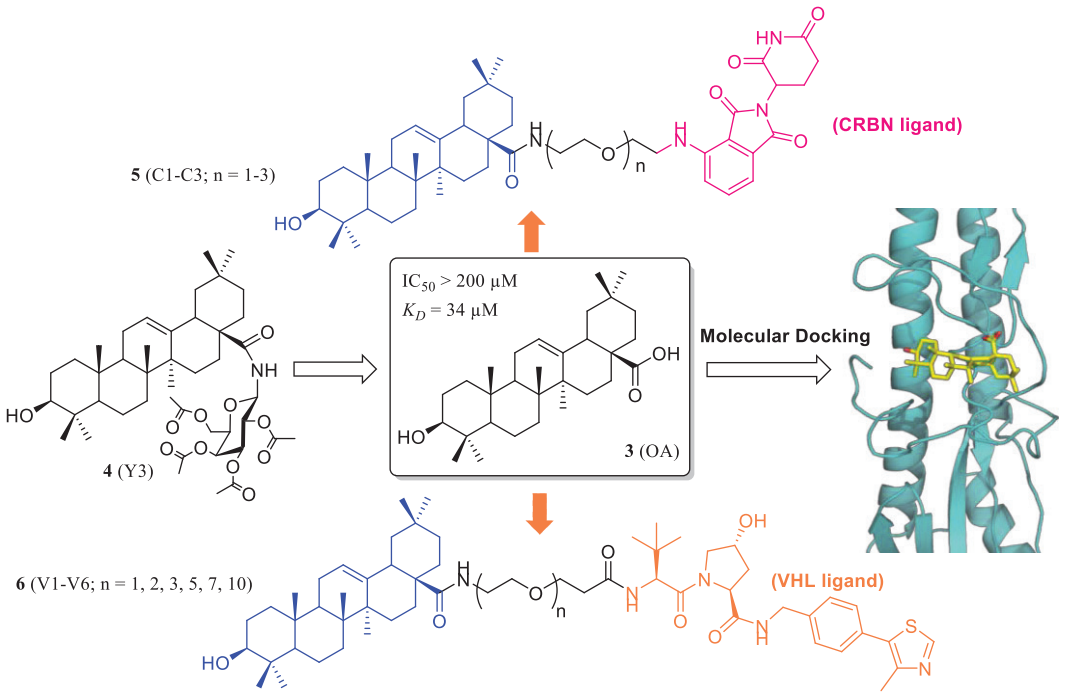

Figure 4 | Design of the HA-targeting degraders. Chemical structures of Y3, OA, CRBN-based PROTACs C1–C3, and VHL-based PROTACs V1–V6 and docking results of OA with influenza HA protein (PDB code: 1RVT), the figure was generated in PyMOL (www.pymol.org).

shown in Western blot assays obtained using treated cell cultures expressing HA, V3-induced HA depletion is avoided in the presence of 30 μmol/L of MG-132. However, the addition of 5 μmol/L of MG-132 has little effect on HA degradation. Immunofluorescence assays have shown that early viral replication is not affected by V3, although HA degradation is observed during virus replication when the virions are released.

Finally, they [67] reported that the OA (i.e., the V3 warhead) tends to bind at inactive sites, as shown by high-resolution mass spectrometry of complexes obtained after photo-crosslinking analysis. These data reveal that V3 acts as an antiviral agent by degrading HA, rather than inhibiting HA function.

Overall, this report not only reports the first case of a PROTAC-based anti-influenza virus application, but extends the potential use of PROTAC technology in antiviral research.

3.2.2 Oseltamivir-based novel PROTACs as degraders targeting influenza virus neuraminidase (NA). Inspired by the successful applications of PROTAC technology, Xu et al. [15] designed and synthesized PROTAC derivatives with different linkers connecting the antiviral drug, oseltamivir, and a VHL ligand. One of the compounds, **7** (Figure 5a), which contains an alkyl chain linker and the E3 ligand, CH_3-VHL, was shown to have an EC_{50} of 0.33 μmol/L against the wild-type influenza virus strain, H1N1, in MDCK cells. Moreover, **7** inhibits viral replication, as determined by measuring the level of viral nucleoprotein (NP) expression. NP expression was shown to be reduced in a dose-dependent manner, and in these experiments the expression was completely suppressed at 1.3 μmol/L, with > 50% degradation at a concentration of 0.31 μmol/L.

The specificity of the observed effects was demonstrated with a negative control compound (**8**, Figure 5a), in which the conformation of the hydroxyl group of hydroxyproline was flipped. As observed in Western blot assays, **8** was unable to degrade target proteins and failed to inhibit viral replication when administered alone, demonstrating that the antiviral activity of **7** is indeed caused by NA degradation. The degradation activity mediated by **7** was significantly inhibited by the addition of ligand-competitive inhibitors, such as oseltamivir, the E3 ligand (CH_3-VHL), or a proteasome inhibitor (MG-132). This study showed that **7** degrades NA in a dose-dependent manner through the ubiquitin-proteasome pathway.

PROTAC technology is particularly interesting due to its ability to overcome drug resistance. Compound **7** was shown to be effective against an oseltamivir-resistant H1N1 strain containing the resistance-associated substitution, H274Y, in the viral NA. It was shown that at concentrations > 1 μmol/L the PROTAC molecule is able to produce a large decrease in NA expression, while oseltamivir had no significant antiviral activity [57]. This result illustrates the promising possibility that PROTAC technology will alleviate the problem of antiviral drug resistance.

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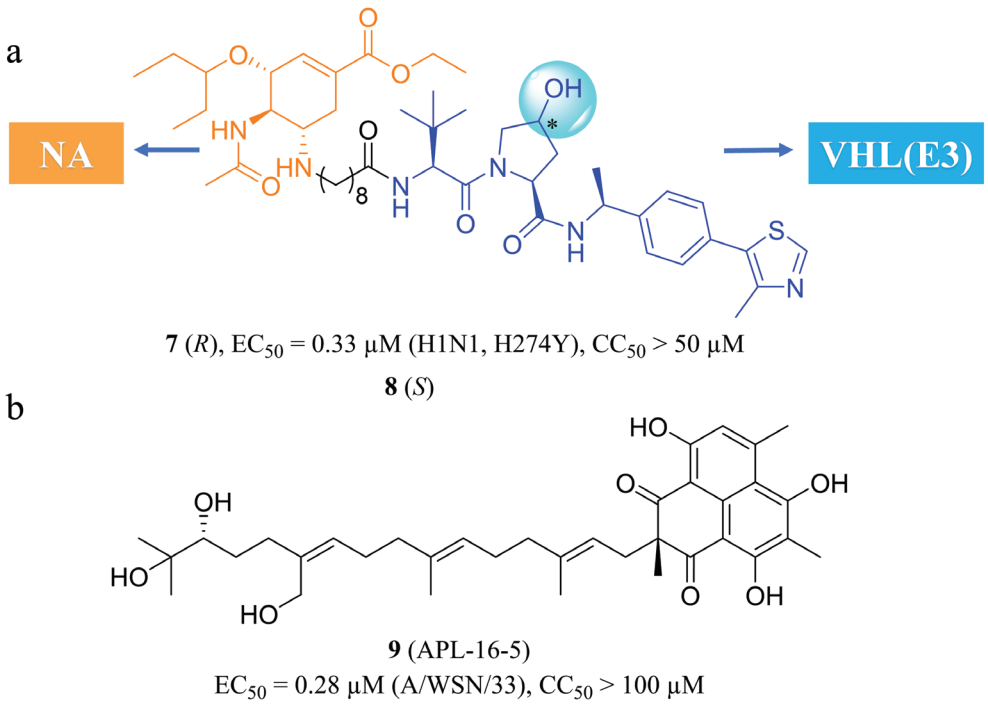

Figure 5 | (a) Chemical structure and inhibitory activities of compound 7 and chemical structure of compound 8. (b) Chemical structure and anti-influenza virus activity of APL-16-5.

3.2.3 An anti-influenza A virus microbial metabolite acts by degrading viral polymerase acidic (PA) endonuclease. APL-16-5 (**9**, Figure 5b) is a metabolite originally isolated from the *Aspergillus* sp., CPCC 400735, an endophytic fungus that grows on plants [70]. APL-16-5, also known as asperphenalenone E, demonstrated potent anti-influenza virus activity in screening microbial metabolites by measuring the inhibitory effects in a system involving infection of the HEK293T-Gluc reporter cell line with the influenza A virus strain, A/WSN/33. APL-16-5 has an EC_{50} value of 0.28 μmol/L [71].

APL-16-5 was found to be a potent antiviral agent targeting *de novo* viral replication. APL-16-5 may act as an antiviral PROTAC that induces proteasome-dependent degradation of the influenza virus RNA-dependent RNA polymerase subunit, PA. The PA subunit has endonuclease activity and cleaves the 5′ region of host mRNA to generate a 10-15 nucleotide 5′ mRNA fragment used as a primer to initiate the synthesis of nascent viral RNA [72]. Interestingly, APL-16-5 induces ubiquitination of PA by engaging the E3 ligase, TRIM25, thereby facilitating the subsequent degradation of the RNA polymerase subunit in the proteasome [71]. APL-16-5 was shown to be an effective antiviral compound in mouse models, thus protecting the animals against lethal influenza A virus infection due to the high degree of homology between human and mouse TRIM25 [44].

3.3 New PROTACs for therapy against HCMV

Human cytomegalovirus (HCMV) is an important pathogen for immunocompromised patients and an etiologic agent of mononucleosis and pneumonia. Drug resistance limits the efficacy of current therapies against HCMV and new drugs and therapeutic strategies are necessary for a better control of the infection [73]. The cyclin-dependent kinase (CDK) inhibitor, SNS032 (**10**, Figure 6), has significant anti-HCMV activity and potential broad-spectrum antiviral activity. THAL-SNS032 (**11**, Figure 6) is a PROTAC molecule based on a CDK9 inhibitor (SNS032) with an EC_{50} of 0.025 μmol/L and a median cytotoxic concentration (CC_{50}) of 0.125 μmol/L (SI of 5.0) in antiviral assays using the HCMV strain AD169-GFP [16]. Interestingly, THAL-SNS032 was shown to be 4-fold more potent than SNS032 in antiviral assays. Studies with different HCMV strains showed that THAL-SNS032 remains effective in the mid-nanomolar concentration range, thus provoking drug-induced degradation of kinases and acting on primary (CDK9) and secondary targets (CDK1, CDK2, and CDK7). Similar results were obtained in antiviral assays using mouse cytomegalovirus as a model. Although THAL-SNS032 was shown to be inactive against varicella zoster virus, human adenovirus, and Zika virus, inhibition of SARS-CoV-2 replication was observed in Caco-2 cells with an EC_{50} value of 0.11 ± 0.02 μmol/L. These data suggest that CDK inhibitors can be used in PROTAC strategies as broad-spectrum inhibitors.

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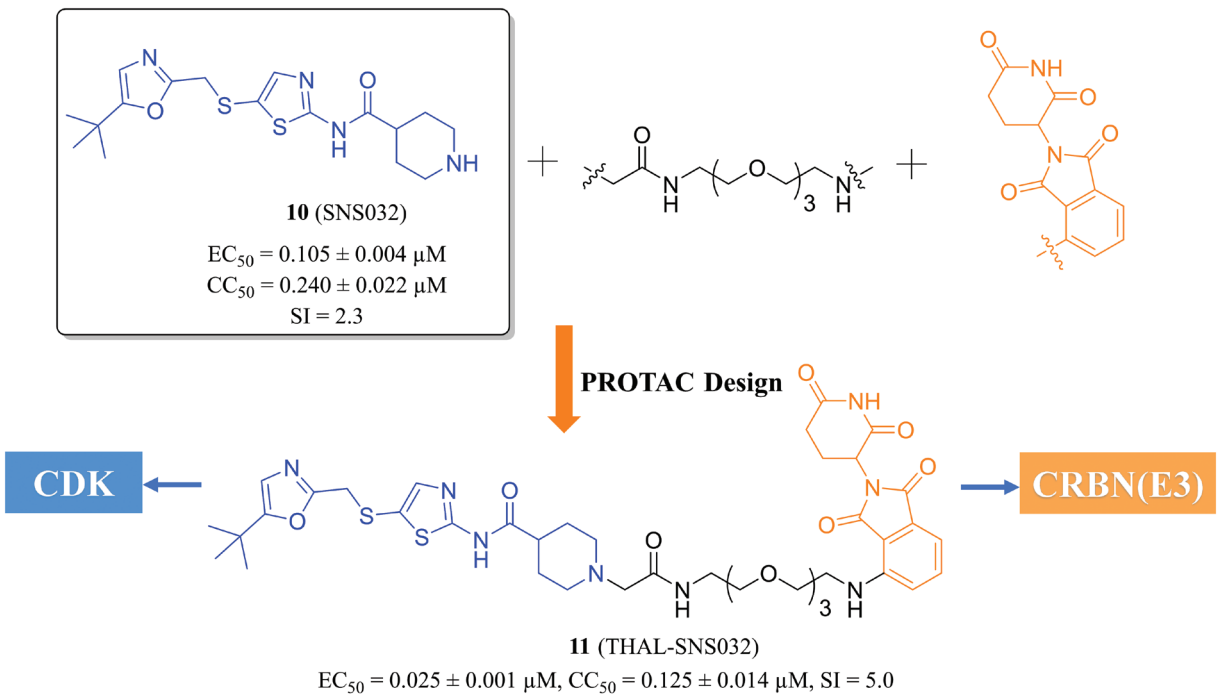

Figure 6 | Chemical structures and anti-HCMV activities in primary human foreskin fibroblasts of SNS032 and THAL-SNS032.

3.4 New PROTACs for therapy against SARS-CoV-2

The 3C-like protease (3CL^{pro}), also known as main protease (M^{pro}), is an important non-structural protein in SARS-COV-2 that has a cleavage site similar to the 3C protease of HCV and other RNA viruses and has an important role in the replication and transcription of coronaviruses [74]. Recently, Panlong Pharmaceutical reported two types of PROTAC molecules [75, 76] based on the 3CL^{pro}

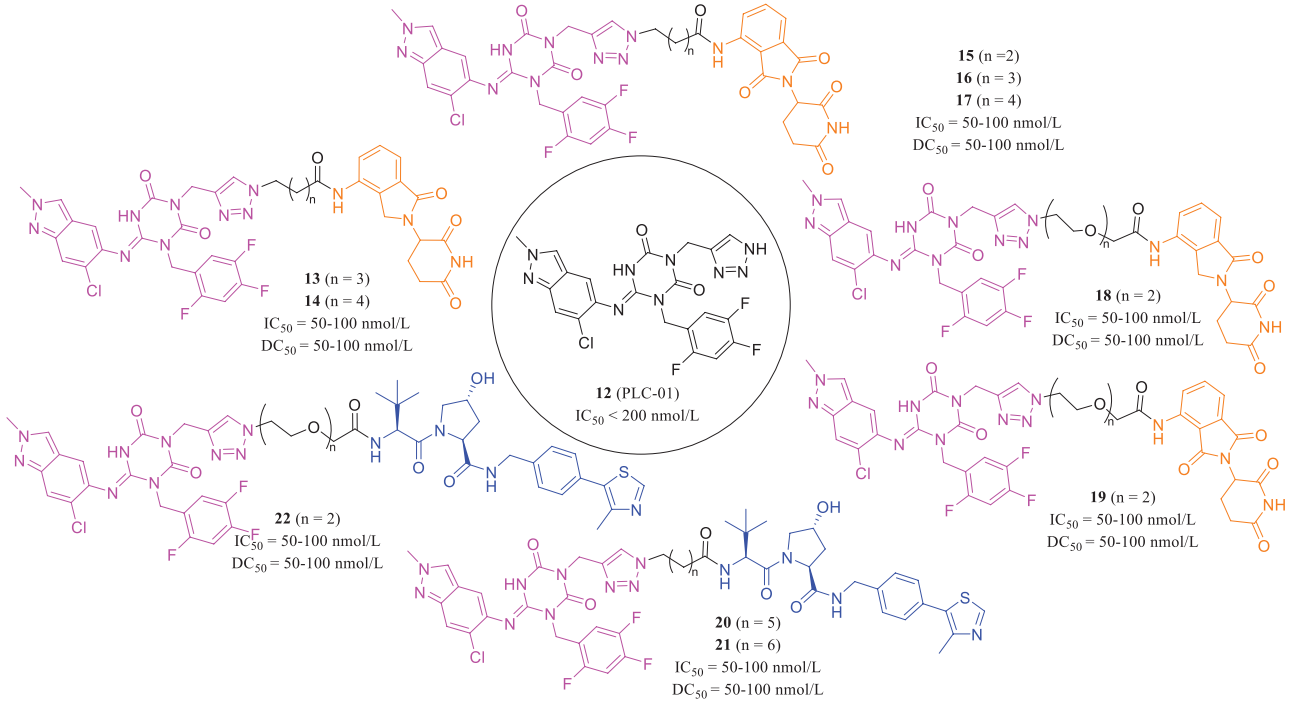

Figure 7 | Chemical structures and targeting-3CL^{pro} activities of PLC-01 and PROTACs.

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candidate drug, PLC-01 (**12**, Figure 7), in two patents. Six series of PROTAC molecules were obtained by combining PLC-01 with the CRBN ligand, thalidomide, and VHL ligands, respectively, using different linkers. Fluorescence resonance and Western blot assays showed that some PROTACs (**13-22**, Figure 7) had inhibitory (IC_{50} = 50-100 nmol/L) and degradation activities (DC_{50} = 50-100 nmol/L) against $3CL^{pro}$ and could be developed as anti-coronavirus degraders. In summary, the successful application of new mechanisms of protein degradation illustrates the great promise of PROTAC in the antiviral field.

4. CONCLUSIONS AND PERSPECTIVES

As an event-driven mode of action, POI degradation is a new avenue for drug discovery and therapeutics, in which PROTAC represents one of the most widely used protein degradation technologies. In this review, we have described the applications of PROTAC in antiviral drug discovery and development, mainly against HCV, influenza virus, HCMV, and SARS-COV-2. To better understand the mechanism underlying PROTAC-induced viral protein degradation, we have summarized the available information on assays focused on the characterization of PROTAC mechanisms of action by analyzing a number of cases mentioned in the text (Figure 8). The choice of characterization assays is essential to optimize molecules and to validate the mechanism of action in the application of the PROTAC strategy. Additionally, cross-validation is necessary because a single assay cannot completely verify the mechanism. We believe that a better understanding and the application of effective characterization assays will facilitate the rational design and mechanism verification of antiviral PROTAC molecules. Therefore, to provide a critical evaluation of the drugs acting through the mechanism used by PROTACs, three "gold standards" were proposed: (i) concentration-dependent degradation of POI; (ii) E3 ligase dependence (methylation of the POI ligand prevents binding of CRBN derivatives); and (iii) proteasome dependence, in which cells treated with MG-132 (proteasome inhibitor) followed by Western blot analysis should demonstrate an effect on POI levels. Clearly, there are other characterization assays that can be used to verify degradation processes, but we envision that the above three standards are the core and most necessary criteria to demonstrate the antiviral effect of selected compounds through a PROTAC mechanism of action.

The relatively high prevalence of different viruses demands for more effective strategies to develop antiviral drugs [10, 77]. A rapidly evolving wave of multi-specific biotherapeutics is indeed imminent [78-80]. PROTACs are multi-specific agents and likely endowed with moderate or high activity after optimization. Theoretically PROTACs, with an event-driven mode of action, could be designed as broad-spectrum agents. Due to the irreversible effects of these drugs and their highly-conserved targets, resistance is less likely to develop. Metalloenzymes are also a large class of antiviral targets, such as HIV integrase, HIV RNase H, and influenza endonuclease. Such metal chelating agents often have the disadvantages of poor membrane permeability

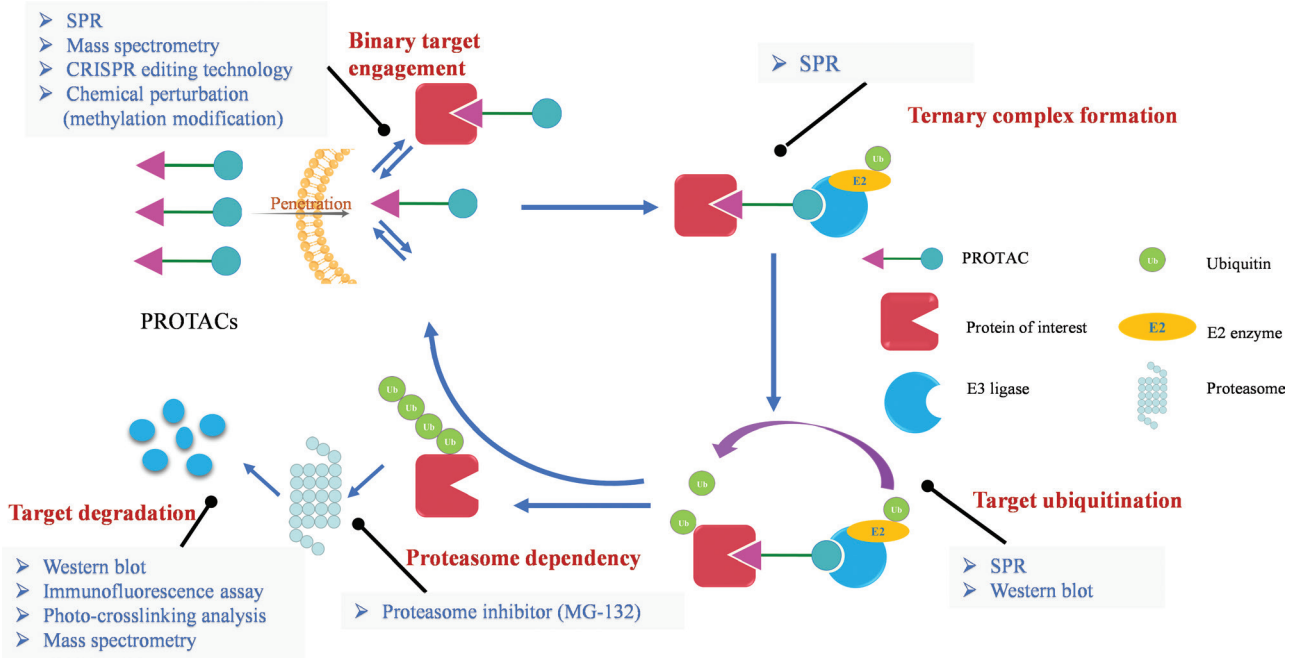

Figure 8 | Assays for characterization of the mechanism of action of PROTACs applied in antiviral field (summarized by this review).

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and poor cell-based antiviral activity [81, 82]. Some metalloenzymes, such as HIV RNase H, have superficial ligand-binding sites and are "undruggable" targets. The above shortcomings are expected to be overcome by designing PROTAC molecules or HyT molecules [83].

From these applications, rational design and compound library screening have become the main strategies for the discovery of existing PROTAC antiviral molecules. In addition, the development of organic synthesis methodologies, and the application of miniaturized and parallelized synthesis technology will greatly facilitate the construction of high-quality PROTAC libraries. The Royal Swedish Academy of Sciences awarded the Nobel Prize for chemistry to Carolyn R. Bertozzi, Morten Meldal, and K. Barry Sharpless in 2022 for the development of click chemistry and bioorthogonal chemistry [84-86]. We envision that the combination of both types of chemistry using combinatorial synthesis and molecular self-assembly systems (dynamic combinatorial chemistry) will provide more rapid, efficient, and reliable approaches for the discovery of bioactive PROTACs [87, 88]. Moreover, developments in structural biology focusing on antiviral drug targets and computational chemistry (e.g., prediction of ternary complexes) will greatly facilitate the design of PROTAC antiviral molecules. Recent advances with a focus on SARS-CoV-2 indicate that computational simulations have a significant role in the design of PROTACs and their potential targets [89-92]. Furthermore, because obtaining a co-crystal structure from experiments is generally very time-consuming, the computational modeling approaches detailed in recent work [93-100], especially the modeling of PROTAC-mediated ternary complexes, could be utilized to design PROTACs for novel targets and optimize PROTACs for current targets so that the many advantages of a PROTAC-based degradation approach can be effectively utilized both rapidly and at reduced cost.

Moreover, a new application of PROTAC-targeted degradation technology for the preparation of influenza vaccines has been recently reported [101, 102]. The vaccine drives specific proteins associated with influenza virus replication to the ubiquitin-proteasome system using PROTAC technology. The modified PROTAC influenza virus vaccine has a reduced ability to replicate autonomously, but the ability to stimulate immune responses is preserved. This finding has undoubtedly been a milestone in the use of PROTAC in vaccine development.

We are aware of the many challenges in developing PROTACs against viral proteins. For example, we need to distinguish between a general antiviral activity (which will eventually reduce/eliminate all viral proteins) and the specific degradation of the target due to the PROTAC mechanism. Furthermore, when using known inhibitors (e.g., telaprevir and oseltamivir) as targeting ligands for antiviral PROTAC development, it is necessary to differentiate the antiviral activity due to target inhibition from that caused by target degradation to determine whether the drug candidate acts through proteasome-mediated degradation.

PROTAC technology still faces many problems and challenges: (i) the high molecular weight of PROTACs; (ii) poor cell permeability; (iii) the relatively small number of available or suitable binders for E3 ligases; (iv) the "hook effect" observed when the concentration of PROTACs is high; (v) the methodologic complexity involved in validating the mechanism of action; and (vi) the potential toxicity of PROTACs, particularly in chronic diseases when long-term use is expected [40, 103, 104]. For these challenges, the drug delivery systems should be improved, while structural biology and computational chemistry could be helpful for optimization purposes [40].

Other methods using protein degradation, such as HyT, molecular glue, and AUTOTAC, can be carried out simultaneously with PROTAC, and compared with each other, are also expected to become an effective method for the development of antiviral drugs [83]. There still remains a large portion of nucleic acids and proteins (such as non-enzymatic and structural proteins) that are undruggable. Inspired by all these developments, the degradation of nucleic acids is also emerging as a new promising strategy in the field of antiviral research [105, 106]. The newly-reported bridged PROTAC strategy could provide a generalizable platform for targeting undruggable viral proteins [107]. Interestingly, protein degradation is not limited to small molecule degraders, and some host proteins can also play a role in degrading viral proteins [108-111].

Finally, PROTAC technology should be considered more than just a weapon to fight pathogenic viruses because it also has broad application prospects in drug target validation. In a very recent study [112], depletion of bromodomain-containing protein 4 (BRD4) by PROTAC degrader resulted in cccDNA inhibition in HBV-infected cells, validating BRD4 as a novel host-based anti-HBV target.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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