CRBN based Nsp13 degrading PROTAC for SARS-CoV-2

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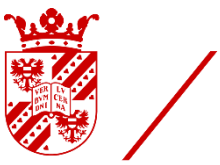

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Contents

Acronyms

+ssRNA: Positive-strand RNA

AIDS: Acquired immunodeficiency syndrome

ARDS: Acute respiratory distress syndrome

CC50: Half-maximal cytotoxic concentration

cIAP: Cellular inhibitor of apoptosis

COVID-19: Coronavirus disease 2019

CRBN: Cereblon

DC50: Half-maximal degradation concentration

DMV: double-membrane vesicles

dsRNA: Double-stranded RNA

E1: Ubiquitin-activating enzyme

E2: Ubiquitin-conjugating enzyme

E3: Ubiquitin ligase

HCMV: Human cytomegalovirus

HDT: host-directed therapy

HECT: Homologous to E6AP C-terminus

HIV: Human immunodeficiency virus

IMiD: Immunomodulatory drug

ITC: Isothermal titration calorimetry

mAb: Monoclonal antibody

MDM2: Mouse double minute 2 homolog

MERS-CoV: Middle East respiratory syndrome-related coronavirus

MOA: Mechanism of action

Nsp: Non-structural protein

POI: Protein of interest

PPI: Protein-protein interaction

PROTAC: Proteolysis targeting chimera

RBR: RING-between-RING family

RING: Really Interesting New Gene

RTC: Replication and transcription complex

SAR: Structure-activity relationship

SARS-CoV: Severe acute respiratory syndrome coronavirus

SF1B: Superfamily 1B

SPR: Surface plasmon resonance

TPD: Targeted protein degradation

UPS: Ubiquitin-proteasome system

VDT: Virus-directed therapy

VHL: Von Hippel-Lindau

ZBD: Zinc-binding domain

Abstract

In the aftermath of the COVID-19 pandemic, the global medical need for effective antivirals that combat SARS-CoV-2 and its new drug-resistant variants is ever important. Proteolysis-targeting chimeras (PROTACs) are an exciting new heterobifunctional drug modality that induces targeted protein degradation of proteins involved in pathology, by exploitation of the ubiquitin-proteasome system (UPS). Using PROTACs as antivirals is a relatively unexplored field in drug discovery. SARS-CoV-2 expresses 16 non-structural proteins (nsps) which facilitate its RNA transcription and replication, virus-host interactions, and host immune modulation functions. Nsp13 helicase is a multi-functional enzyme with the main function being the nucleotide triphosphate (NTP)-dependent unwinding double-stranded RNA (dsRNA) in the 5' to 3' direction. Nsp13 helicase is a highly conserved, essential enzyme needed for SARS-CoV-2 transcription, replication, and ultimately its survival. This essay presents the theoretical design of a nsp13 helicase degrading PROTAC based on CRBN and the non-competitive inhibitor of nsp13, SSYA010-001.

Introduction

Viruses are noncellular, infectious, obligate parasites consisting of a genome surrounded by a capsid coating, with some viruses having an additional host-derived bilayer envelope[1]. Depending on the virus, the genome consists of RNA or DNA, is either double- or single-stranded, positive- or negative sense, along with many other variations[1]. A virus infects host cells and uses its cellular apparatus to express proteins for further replication, immune evasion, and host infection[1]. In the process, the host cell is often harmed or killed, causing detrimental consequences for the host.

Viral infections have historically had an enormous impact on global health. Smallpox, caused by the variola virus, has caused an estimated 400 million deaths in the 20th century[2]. In 1981, the human immunodeficiency virus (HIV) caused the acquired immunodeficiency syndrome (AIDS) epidemic to infect over 75 million people and claim over 32 million lives[3]. The past two decades have seen the rise of coronaviruses which are enveloped, positive-sense single-stranded RNA viruses[4]. Coronaviruses are spread via respiratory aerosols and droplets and induce flu-like symptoms in the host[1]. Severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) broke out in 2002 and infected over 8000 people internationally[5]. In 2012, Middle East respiratory syndrome-related coronavirus (MERS-CoV) caused the spread of MERS, resulting in a reported 2080 infections and 722 deaths[5]. Recently in late 2019, the coronavirus disease 2019 (COVID-19) pandemic saw an unprecedented global undertaking to reduce the spread of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[4]. COVID-19 ranges from mild flu-like symptoms to severe organ impairment or death[6]. The reported amount of cases was

a staggering 776 million infections with over 7.0 million deaths[7]. As new viral epidemics continue to emerge, the need for effective vaccines and antivirals is paramount.

Vaccinations are a powerful drug modality that reduces host transmission, alleviates serious complications, and reduces the likelihood of infection[4]. Smallpox, for example, has been completely eradicated using vaccines, with no reported cases since 1978[2]. Vaccines have their limitations and need complementary antiviral drugs for a broader antiviral strategy. For one, vaccines rely on the adaptive immunity of the host which can be compromised by pharmacological immunomodulation or by risk factors such as age, metabolic disorders, and autoimmune disease[8]. The rapid emergence of mutated variants of viruses reduces the efficacy of vaccines, such as the Omicron variant of SARS-CoV-2[1].

The last two decades have seen the rise of proteolysis-targeting chimeras (PROTACs), a new heterobifunctional drug modality that directs the endogenous ubiquitin-proteasome system (UPS) to selectively degrade proteins involved in disease. Not dependent on binding active sites, PROTACs can degrade proteins without enzymatic/receptor functionality. With viruses, PROTACs can be used to target both host- and viral proteins, allowing for two strategies to combat the virus, host-directed and virus-directed[9].

PROTACs can therefore be an exciting therapeutic prospect that can degrade "undruggable" proteins in diseases that were previously considered hard to treat.

PROTACs

Modern drug discovery has traditionally relied on drug modalities such as small-molecule inhibitors. Small molecule inhibitors are designed to occupy the active site of a target protein and must compete with endogenous substrates[10]. Target proteins have direct involvement in the pathology of disease such as ion channels, kinases, and receptors[10]. While effective, small molecule inhibitors suffer from developing off-target effects and drug resistance with long-term usage[11]. Additionally, reliance on binding an active site renders many proteins "undruggable" using small molecule inhibitors.

PROTACs have a broader range of targets relative to small molecule inhibitors due to their unique event-driven mechanism of action (MOA)[10]. A PROTAC consists of three conceptual parts. A "anchor" ligand that binds an E3 ubiquitin ligase, a "warhead" ligand that binds a protein of interest (POI), and a linker connecting the two[1]. A PROTAC can bind the two respective ligands, forming the POI-PROTAC-E3 ternary complex (**Figure 1**). Bringing the POI and the E3-E2 close to each other catalyzes ubiquitin transfer from E2 to the POI which happens multiple times until a polyubiquitin chain is formed[12]. The polyubiquitin-tagged POI is then recognized and bound by the 26S proteasome and

degraded[12]. In essence, PROTACs can steer the naturally occurring proteolytic apparatus of the cell toward degrading medically relevant proteins.

Once ubiquitination is finished, the complex dissociates, leaving the PROTAC to repeat the MOA[12]. PROTACs can therefore be effective at substoichiometric concentrations compared to the POI, resulting in lower doses needed and longer dosing intervals[11]. PROTACs also fully degrade a protein, negating all enzymatic and non-enzymatic functionality from the protein[12].

PROTACs do not require active site binding for their function[10]. This allows targeted protein degradation (TPD) of previously "undruggable" targets such as scaffolding proteins, transcription factors, and proteins that rely on protein-protein interactions (PPIs)[10]. Moreover, drug-resistant isoforms of proteins with mutated active sites can still be degraded by PROTACs[10]. TPD of mutant proteins is especially useful in diseases with resistance to occupancy-driven inhibition e.g. multi-drug resistant bacterial infections[10].

PROTACs are a relatively new drug modality that is being explored for indications such as cancers, neurodegenerative diseases, bacterial infections, auto-immune diseases, cardiovascular diseases, and viral infections[13].

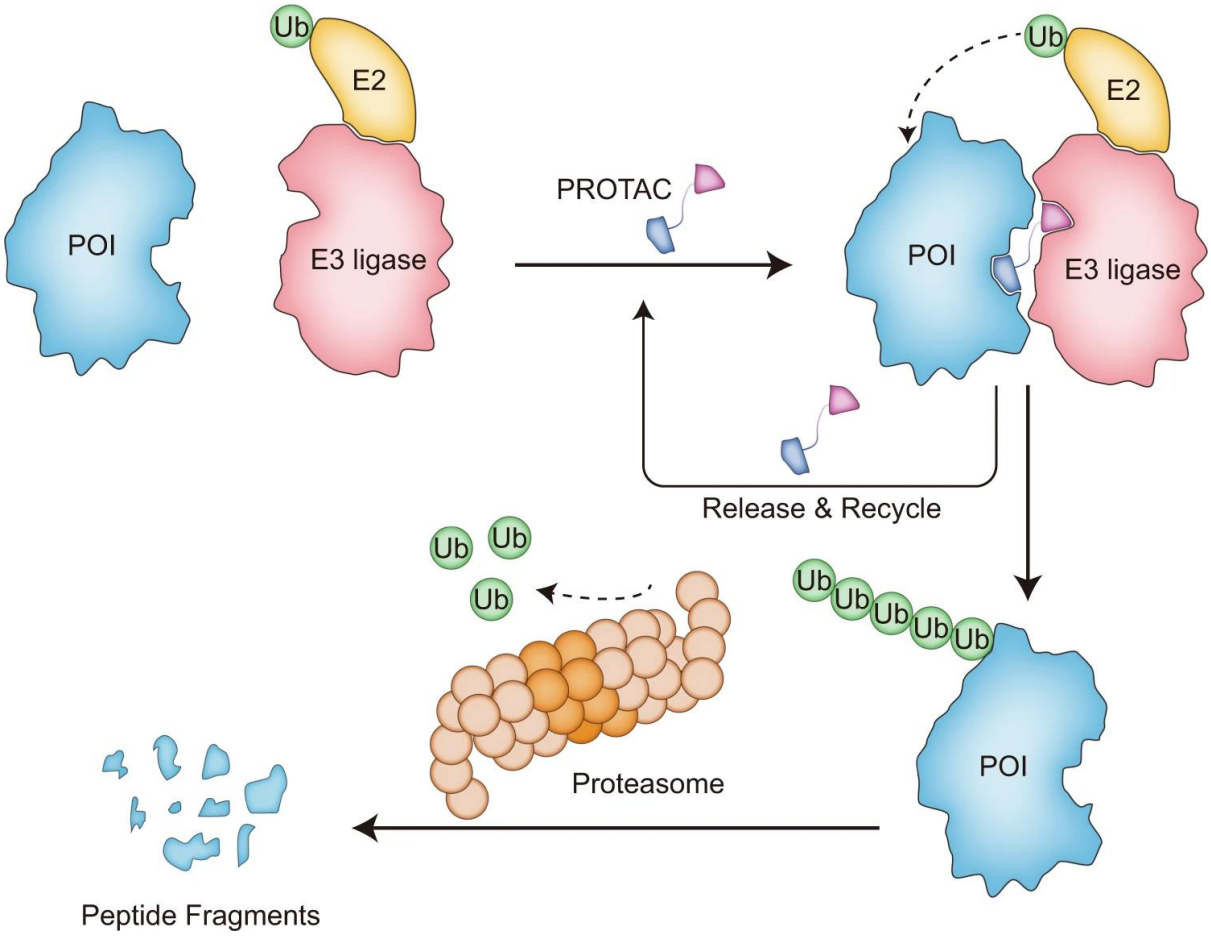

Figure 1 The mechanism of action of PROTAC-induced protein degradation by use of the UPS[14].

Structure-activity relationship

PROTAC Anchor

The ability of a PROTAC to selectively and effectively degrade a protein relies on the anchor, the warhead, the linker, and the stability of the ternary complex. Optimization of any individual PROTAC component may impact the behavior of the other PROTAC components, ultimately leading to a complicated structure-activity relationship (SAR)[10].

The E3 ligase ligand is important as it influences the degradation profile of a PROTAC as well as the pharmacokinetic properties[15]. The human proteome contains three types of E3 ligases namely the Really Interesting New Gene (RING) family, the Homologous to E6AP C-terminus (HECT) family, and the RING-between-RING family (RBR)[13]. The RING family of E3 ligases transfers ubiquitin directly from E2 to a POI, while the HECT and RBR E3 ligases first form a thioester intermediate preceding ubiquitin transfer[13].

The RING family contains over 600 E3 ligases to be targeted, of which, the majority has been unexplored for PROTAC design[15]. Commonly targeted E3 ligases are from the RING family namely cereblon (CRBN) and von Hippel-Lindau (VHL)[12]. Targeting these ligases is desirable due to their ubiquitous expression in most cell types, allowing for broad PROTAC application[13]. Both of these ligases also have well-established structures, functions, available high-binding affinity ligands, and PROTACs which successfully incorporated them[13].

A preferable E3 ligase will be able to engage in a spatial relationship with the POI that is conducive to a degradation-competent ternary complex[12]. Additionally, to form a ternary complex, the E3 ligase must be localized in the same cellular compartments as the POI[12]. Subcellular co-localization can be accomplished by choosing a ubiquitous E3 ligase or an E3 ligase that is tissue-specific for the targeted pathology[12]. Other considerations are whether an E3 ligase active sites are prone to mutations and if the enzyme contains structural 'on' or 'off' mode[12].

The first iterations of PROTAC anchors consisted of peptide-based ligands that were large in molecular size[16,17]. Due to low cell permeability, the switch was made from peptide ligands to covalent small molecule E3 ligase recruiters[13]. The small molecule recruiters targeted mouse double minute 2 homolog (MDM2), cellular inhibitor of apoptosis (cIAP), CRBN, and VHL[13].

PROTAC ligands for CRBN and VHL are well-established in terms of their binding affinity, potency, and specificity[18]. Furthermore, the established ligands possess favorable physicochemical properties such as higher cell permeability, lower molecular weight, relative inertness, and increased aqueous solubility[18]. PROTACs with CRBN and VHL ligands possess higher degradation efficiency (DC_{50}/DC_{max}) compared to PROTACs with other explored anchors[12].

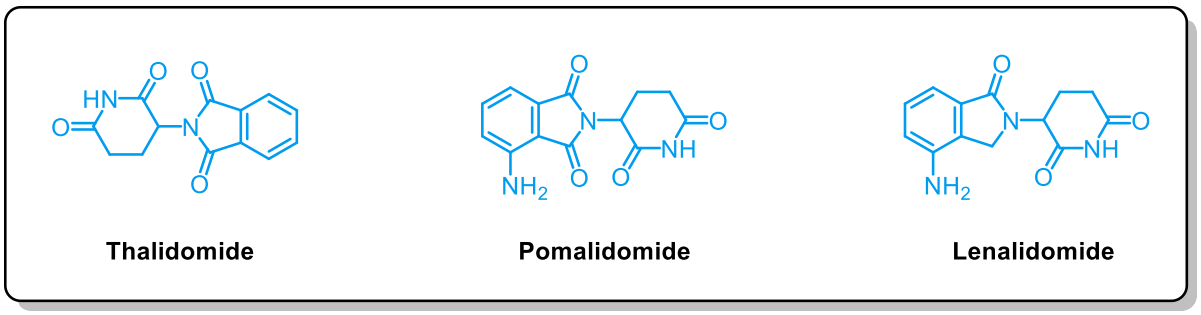

Figure 2 The chemical structures of CRBN E3 ligase ligands thalidomide, lenalidomide and lenalidomide.

CRBN is one of the substrate receptors of the Cullin-Ring ligase 4 E3 ubiquitin ligase complex ($CRL4^{CRBN}$). The complex consists of four domains: the substrate receptor CRBN, a RING finger domain protein (Roc1 or RBX1), Cullin 4 (CUL4) scaffold protein damaged DNA binding protein 1 (DDB1)[19]. In normal physiological conditions, $CRL4^{CRBN}$ facilitates the degradation of transcription factors (IKZF family) involved in neuronal excitation, immunomodulation, energy metabolism, cell proliferation, and apoptosis[19]. $CRL4^{CRBN}$ also bind glutarimide moieties with high affinity, these are found in the immunomodulatory drug (IMiD) thalidomide and its analogues pomalidomide and lenalidomide (**Figure 2**)[19]. IMiDs alter substrate recognition of $CRL4^{CRBN}$, allowing ubiquitination and proteasomal degradation of neosubstrates[19]. IMiDs have a high binding affinity for CRBN and are thus frequently incorporated into PROTACs as the CRBN E3 ligase ligand[19].

PROTACs incorporating CRBN E3 ligase ligands have reached clinical trials with promising results, namely ARV-110 (NCT03888612) and ARV-471(NCT04072952) (**Figure 2**)[20,21]. ARV-110 is currently in phase II trials and is designed for the indication of metastatic castration-resistant prostate cancer, showing efficient degradation of androgen receptors (DC_{50} = 1 nM) in xenograft murine models[21]. ARV-471 is indicated for metastatic breast cancer and recently entered phase III trials due to its efficient degradation of treatment-resistant estrogen receptors[20,22].

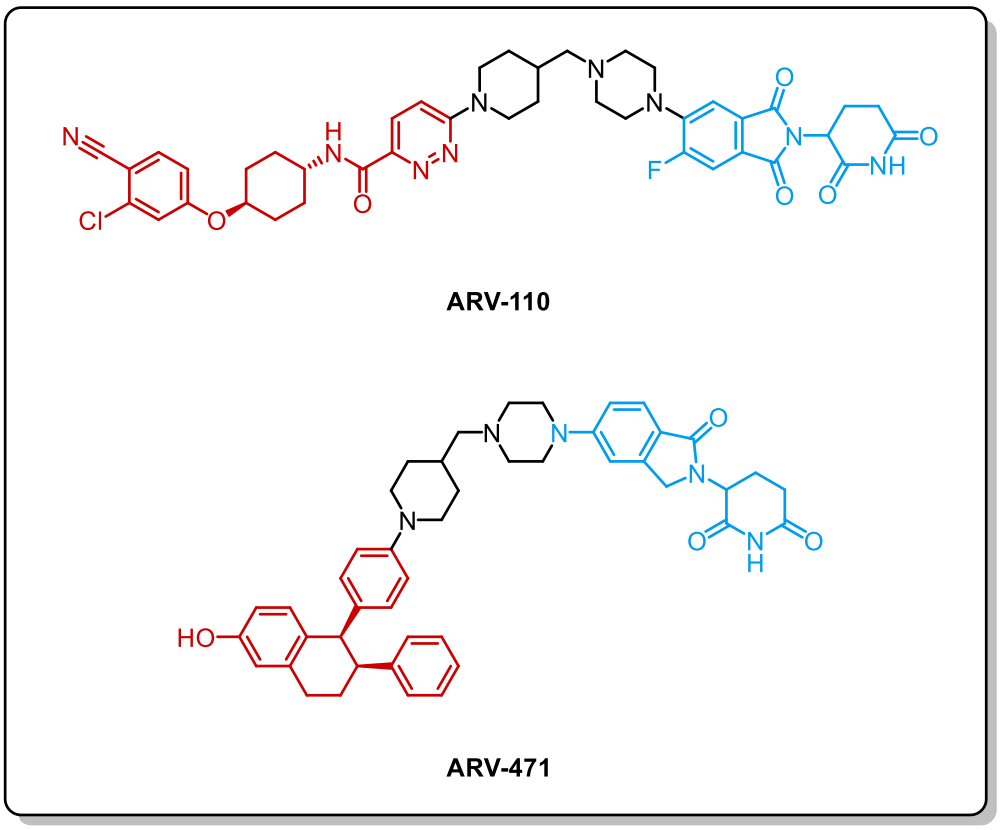

Figure 3 The chemical structures of CRBN-based PROTACs ARV-110 (top) and ARV-471 (bottom). Respective anchors are indicated in blue, linkers in black, and warheads in red.

VHL is the substrate recognition receptor of the Cullin-2 RING ligase complex ($CRL2^{VHL}$)[23]. The complex is comprised of the subunits VHL, ELoB, EloC, Rbx, and CUL2[23]. CRL^{VHL} exhibits E3 ligase activity for several substrates, most notably it facilitates the polyubiquitination of the transcription factor hypoxia-inducible factor-1α (HIF-1α)[23]. Subsequent degradation of HIF-1α downregulates vascular endothelial growth factor (VEGF) thus reducing angiogenesis[12]. Modern iterations of VHL ligands are based on VH032 and its broad range of derivatives (**Figure 3**)[12]. Although VHL-based PROTACs are the most commonly cited in literature, they suffer from compromised ternary complex formation resulting in lower TPD efficiency[23]. DT2216 is a VHL-based B-cell lymphoma-extra large (Bcl-xL) degrader currently in phase I of clinical trials for the indication of plasma cell leukemia (**Figure 4**)[24].

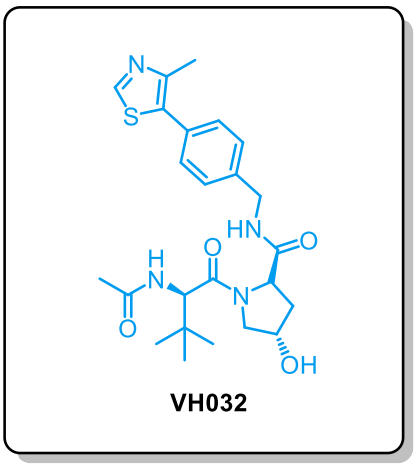

Figure 4 The chemical structure of VHL E3 ligase ligand VH032.

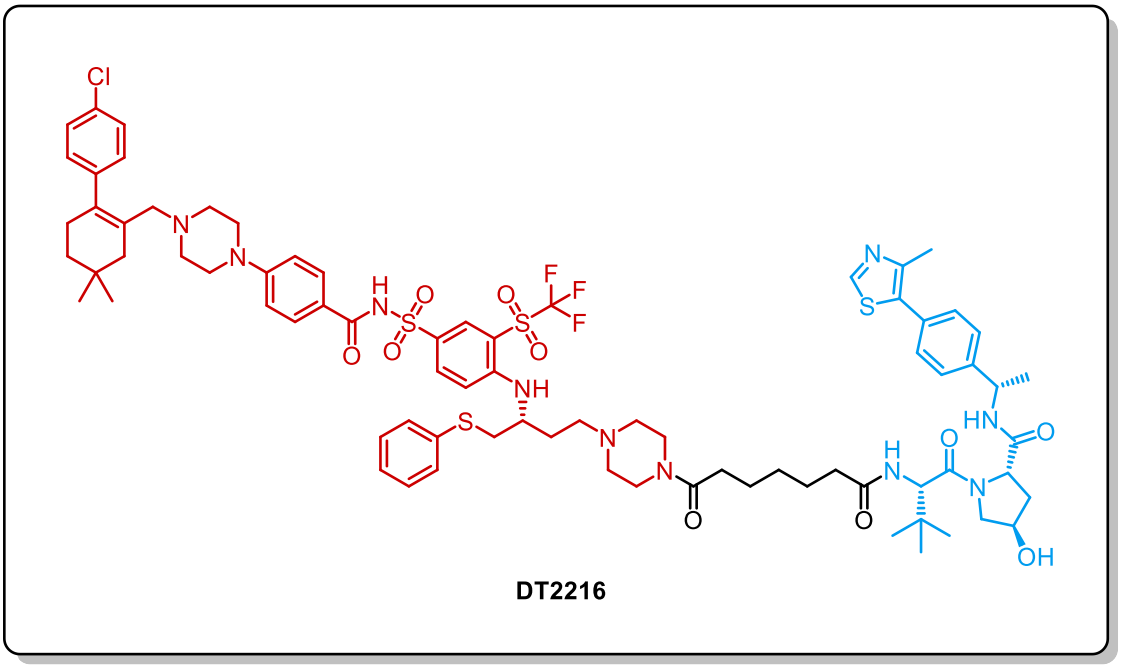

Figure 5 The chemical structure of the VHL-based PROTAC DT2216. It is comprised of the VH032 anchor (blue), alkyl linker (black), and the Bcl-xL binding ligand (red).

PROTAC Warhead

The warhead of the PROTAC is typically an approved small molecule inhibitor of the POI[12]. Desirable warheads bind the POI with high affinity and specificity[12]. A POI ligand can also influence the ternary complex formation and the physicochemical properties of the PROTAC[12]. These two factors have downstream effects on the proteolytic efficiency and pharmacokinetic properties of the PROTAC[12].

PROTAC Linker

The linker of a PROTAC contributes significantly to the selectivity and ability to degrade a target, however, the structure-activity relationship (SAR) predictions of a linker remain difficult. Linkers can vary in length, functional group configuration, and the attachment

site to the two ligands[25]. Linkers also vary in their flexibility, with some PROTACs having higher degradation efficiency with rigid linkers and others with flexible linkers[25].

The structure of a linker influences the binding affinity of the ligands to their targets and the spatial relation of the ternary complex. An effective linker ensures stable ternary complex formation whilst allowing for ubiquitin transfer from E2 to the lysine residues of the POI[25]. Aside from protein degradation, a linker also affects pharmacokinetic parameters such as aqueous solubility and cell permeability[25].

The complicated SAR of linkers makes them difficult to rationally design. Systematic testing of different linkers is often required to obtain the optimal linker[25]. Linker 'toolboxes' are available depending on the E3 ligase used. Steinebach et al. (2019) proposed a toolbox for CRBN-based PROTAC linkers[26]. The toolbox contains established linker compositions that differ in their lengths, functional groups, and physicochemical properties, allowing previously validated linkers to be applied to new PROTAC designs[26].

Ternary complex

Another important factor to consider when designing PROTACs is the stability of the ternary complex[27]. Ternary complex stability has been regarded as more important to the degradation capability of a PROTAC than individual ligand binding affinities[27]. A proper ternary complex ensures all ligands are bound and spatially aligned in a way where polyubiquitination of the POI is possible[27]. When tagging is complete, a desirable ternary complex will dissociate so the POI can be degraded and the PROTAC can repeat its MOA[10].

Complex stability is determined by PPIs between the warhead and the anchor, steric hindrance, and the composition of the linker[10]. PPIs between the E3 ligase and the POI surfaces can be thermodynamically attracting or repulsing[28]. The ratio between attractive and repulsive forces is measured in the unit cooperativity (α) (**Figure 6**)[29]. A greater number of attracting PPIs causes positive cooperativity ($\alpha > 1$), which increases the thermodynamic stability of the ternary complex. The inverse is true of repulsive PPIs[29].

Each ternary complex has an individual "Goldilocks zone" of cooperativity that depends on the E3 ligase, the POI, and all three moieties of the PROTAC. Effective degraders have been designed with a variety of cooperativity values, including both positive and negative[29]. An excess of repulsive PPIs can prevent any complex formation and excessive attracting PPIs can form a compressed complex that is degradation-incompetent[12]. While the consensus is in flux, positive cooperativity contributes more to an efficient degradation profile due to the negation of the hook effect[29].

The hook effect is a phenomenon that arises when the concentration of a PROTAC is too high[29]. PROTACs saturate the binding sites on the E3 ligase and the POI, resulting in the formation of binary complexes only that prevent protein degradation (**Figure 6**)[10]. The

hook effect can be mitigated by reducing the PROTAC concentration and by ensuring a ternary complex with positive cooperativity[10].

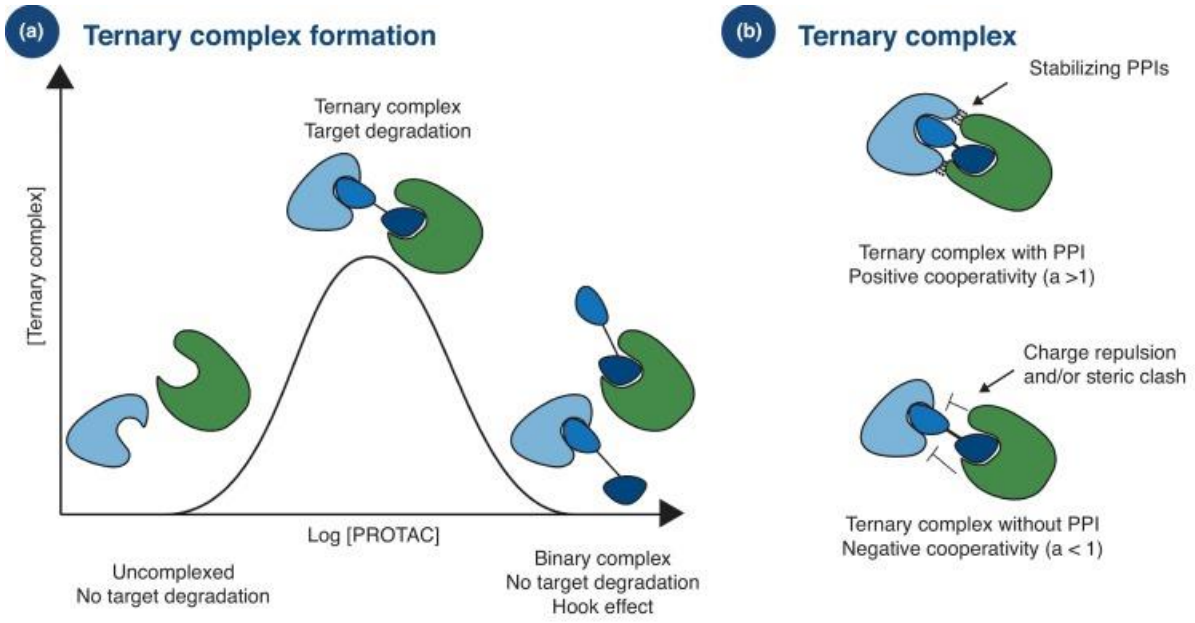

Figure 6 (a) Ternary complex formation graph with log [PROTAC] concentration on the x-axis and [ternary complex] concentration on the y-axis. The Hook effect is seen with a high [PROTAC] resulting in binary complex formation and no TPD. (b) Ternary complex cooperativity[10].

In silico models are used to predict ternary complex stability by modeling repulsing and attracting PPIs between E3 and the POI[25,29–31]. Biochemical assays are used to quantify and validate ternary complex formation using surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC)[25].

PROTAC Pharmacokinetics

Despite the promising efficiency of PROTACs, they face several pharmacokinetic challenges. PROTACs do not adhere to any of Lipinski's rule of 5[23]. The PROTACs have high molecular weight, low aqueous solubility, many hydrogen bond donors and acceptors[23]. These physicochemical properties negatively influence the overall ADME characteristics[32]. Moreover, degraders also suffer from low cell permeability that causes reduced uptake in the gastrointestinal tract[32]. With oral administration, PROTACs are likely to be oxidized when hepatically cleared, further reducing the bioavailability[32].

Pharmacokinetic issues can be slightly mitigated by adjusting the chemical structure of the PROTAC to increase aqueous solubility and cell permeability[32]. Other solutions include the use of nanoformulations to increase bioavailability and to selectively target a tissue of interest[32].

SARS-CoV-2

In late 2019, the zoonosis coronavirus disease 2019 (COVID-19) caused an unparalleled global pandemic with a confirmed 6.9 million deaths[4]. The causal severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) that hailed from Wuhan, China, spread worldwide via respiratory aerosols and droplets resulting in 768 million confirmed infections[4]. International efforts were made to research SARS-CoV-2 pathology and develop therapeutic interventions. While preventative vaccines and antivirals reduced transmission, the rapid emergence of SARS-CoV-2 variants negated the efficacy of these drugs[4]. After the pandemic, the discovery of SARS-CoV-2 antivirals is needed to develop a targeted therapy and keep further pandemics at bay.

SARS-CoV-2 is a positive sense, enveloped, single-stranded RNA virus of the *Coronaviridae* family[4]. SARS-CoV-2 is a betacoronavirus that can infect humans, resulting in the respiratory illness Coronavirus disease (COVID-19). Symptoms range from common cold-like symptoms to severe organ injury and death[4]. The virus has four human variants in circulation with zoonotic origin. The alphacoronavirus variants (HCoV-229E and HCoV-NL63) are derived from bats, while the two betacoronaviruses (HCoV-OC43 and HCoV-HKU1) infected humans via rodents and cattle[33].

Mild infections with SARS-CoV-2 induce flu-like symptoms such as cough, fever, diarrhea, myalgia and headache[6]. If the virus is not cleared by the innate or adaptive immune system, the disease can progress to potentially lethal acute respiratory distress syndrome (ARDS) or extrapulmonary diseases[6].

Virion structure

The SARS-CoV-2 virion consists of envelope proteins (E), spike glycoproteins (S), nucleocapsid protein (N), membrane proteins, a host-derived lipid bilayer, and within the virion resides the single-stranded RNA genome (+ssRNA) (**Figure 7**)[6]. The structural proteins facilitate viral packaging, budding and assembly of new virions[4]. Additionally, the virion expresses essential non-structural proteins (nsps) for viral replication and translation[6]. Complementary non-essential accessory proteins are also encoded which modulate the virus' immuno-evasive and host-virus interactions[6].

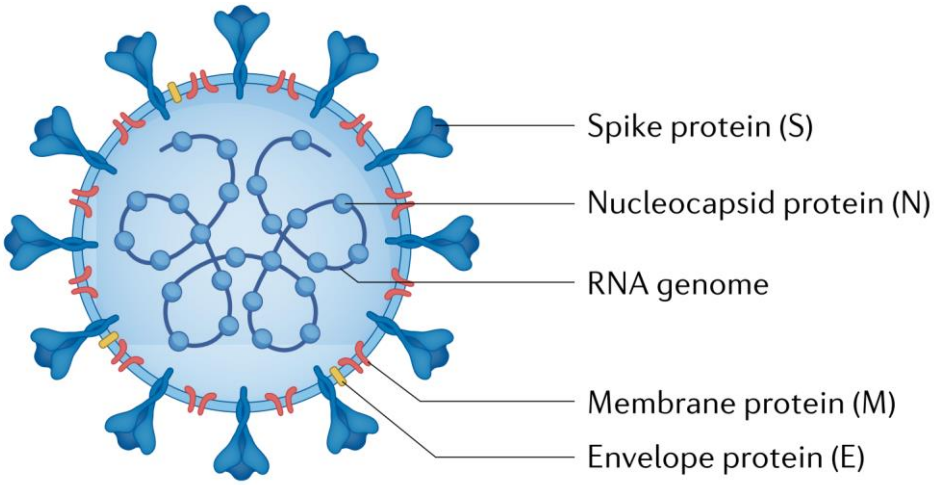

Figure 7 The virion structure of the SARS-CoV-2 virion[6].

Viral Entry

The spike protein is a class I viral fusion glycoprotein that is responsible for viral entry of a host cell[34]. The protein is located on the virion envelope and is arranged in a homotrimer conformation. The spike protein can be subdivided into four components, the S1 subunit, the S2 subunit, the polybasic S1-S2 boundary (or PRRAR), and the furin motif (S2') located near the S1-S2 boundary. Viral entry can occur via two mechanisms namely cell surface entry or endosomal entry.

The S1-S2 boundary can be cleaved before entry mechanisms by furin proteases of infected producer cells[4]. The spike protein is then primed, increasing affinity for ACE2 and consequently amplifying tropism[4].

Cell surface entry starts with the binding of S1 to the angiotensin-converting enzyme 2 (ACE2) receptor (**Figure 8**)[34]. S1 then undergoes a conformational change that exposes the S2' motif on the S2 subunit. The enzyme transmembrane serine protease 2 (TMPRSS2), which is required for this entry mechanism, cleaves the S2' motif[34]. This has two outcomes, the S2' fusion peptide is exposed and the S1 subunit sheds from S2[34]. The S2' fusion peptide then binds the host cell membrane, allowing this membrane to fuse with the virion membrane. A fusion pore is formed, through which, +ssRNA is released into the cytosol of the host cell. Here, the +ssRNA is uncoated, replicated, and translated[34].

Endosomal cell entry also starts with S1 binding ACE2, resulting in S2' being exposed (**Figure 8**)[34]. In tissues where ACE2 or TMPRSS2 expression levels are low, host-cell clathrin facilitates endocytosis of the virion into an endolysosome. In the acidic endolysosome, cathepsin L or cathepsin B cleave S2', revealing the fusion protein. Then the aforementioned membrane fusion occurs between the virion and the endolysosome bilayer of the host. +ssRNA is released, uncoated and translated into the host cytosol.

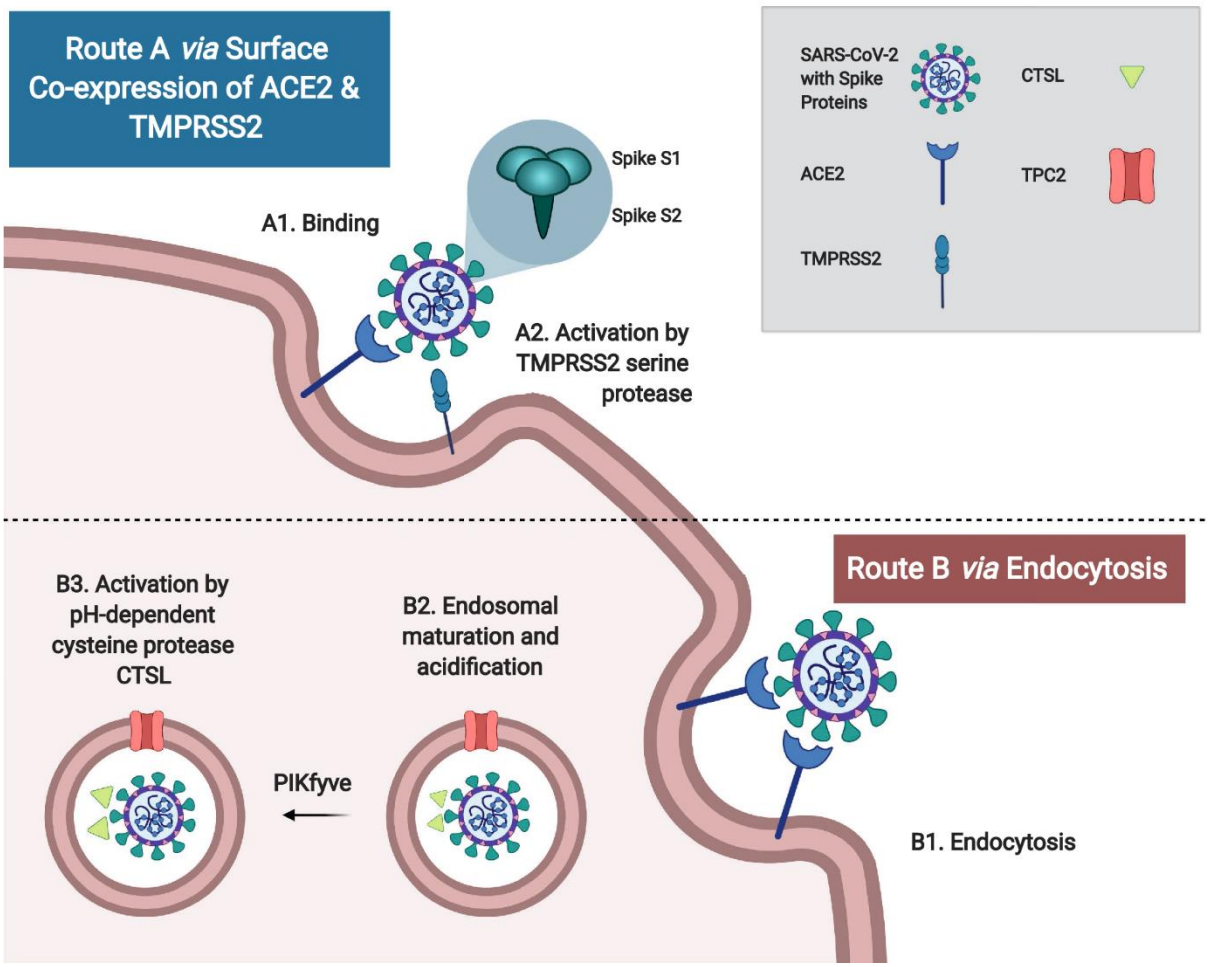

Figure 8 The viral entry mechanism of SARS-CoV-2. Route A) Cell surface entry facilitated by ACE2 and TMPRSS2. Route B) Endosomal cell entry facilitated by clathrin-mediated endocytosis[35].

Non-structural proteins

The +ssRNA is circa 29.9 kb long and contains a 5' cap and a polyadenylated 3' cap[4]. From the 5' cap onwards, the first two-thirds of the viral genome consist of open reading frames 1a and 1b (ORF1a and ORF1b) (**Figure 9**)[4]. ORF1a and ORF1b translation produces two polyproteins 1a and 1b (pp1a and pp1b). Pp1a and pp1b are cleaved by viral cysteine proteases papain-like protease (nsp3/PL^{pro}) and main protease (nsp5/M^{pro}) into 16 different nsps[4]. Pp1a cleavage results in nsp1 to nsp11 and ppa1b cleavage results in nsp1 to nsp10 and nsp12 to nsp16[4]. The resulting 16 nsps are involved in RNA replication, transcription, and virus-host interactions with the host cell[4].

The last third of the genome after ORF1b until the 3' cap, encodes for four structural proteins namely the spike protein, the nucleocapsid protein, the envelope protein, and the membrane protein[4]. An additional nine accessory proteins with immune-evasive functions are also encoded[4]. In an infectious cycle, sg mRNA translates this part of the genome, resulting in the expression of the structural and accessory proteins[4].

Nsp1 binds the host's ribosome and blocks translation in addition to suppression of IFN production[4]. Nsp2 until nsp16 are all involved in forming the viral replication and transcription complex (RTC) that facilitates RNA synthesis[4]. Nsp12, nsp13, nsp14, and nsp16 are essential for RNA synthesis, while the other enzymes of the RTC are co-factors and immuno-modulators. The RTC forms double-membrane vesicles (DVMs) out of endoplasmic reticulum membranes, where gRNA is replicated and sg mRNA is transcribed[4]. RNA leaves the DMVs, after which they are translated into structural and accessory proteins[4]. At the ER-Golgi intermediate compartment, structural and accessory proteins form the nucleocapsid, and the envelope of the virus, preluding virion maturation

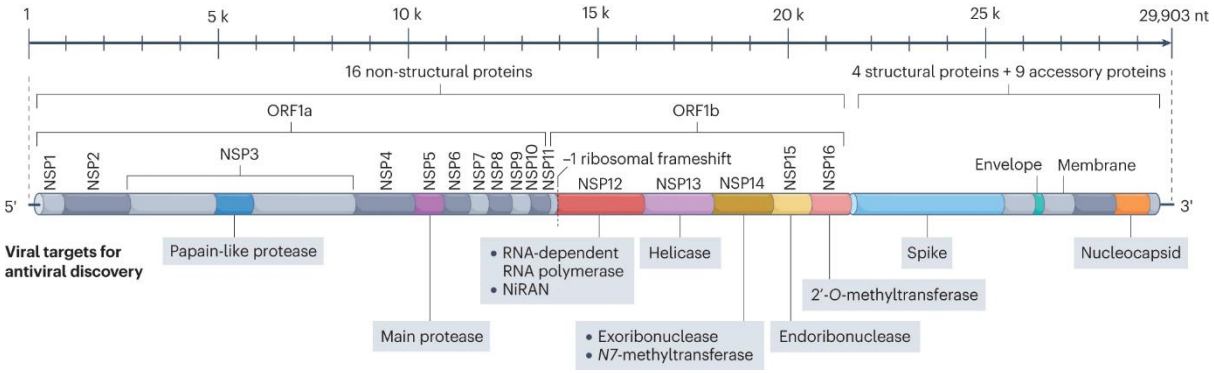

Figure 9 The SARS-CoV-2 positive-sense, single-stranded RNA genome encoding non-structural proteins, structural proteins, and accessory proteins[36].

SARS-CoV-2 hijacks the UPS to weaken host immune responses, accelerate viral entry, and prevent proteolysis of viral proteins[37]. Nucleocapsid proteins and nsp3 (PL^{pro}) both inhibit IFN response by the host, thus reducing the immune response to the viral infection. Nsp3 in particular has deubiquitinating enzyme functionality which prevents proteolysis of viral proteins and promotes the deactivation of IFN agonizing factors such as IRF3 and ISG15[37]. Nucleocapsid proteins impair RIG1 activation, resulting in downstream IFN antagonization, further reducing host immune responses[4].

Nsp13 helicase

Nsp13 helicase is 601 amino acids long, a multi-functional enzyme of the helicase superfamily 1B (SF1B). This essential enzyme plays a key role in unwinding and separating double-stranded RNA for the purpose of RNA transcription and replication[38].

Nsp2 up until nsp16 set up the viral replication and transcription complex (RCT) together to synthesize viral messenger, genomic, and sub-genomic RNA[4,39]. To replicate, transcribe, proofread, and modify RNA, the essential nsps are nsp12, nsp13 helicase, nsp14, and nsp16[4]. Nsp12 is the RNA-dependent RNA polymerase (RdRp), which conjugates with cofactors nsp7 and nsp8 for increased polymerase activity[4].

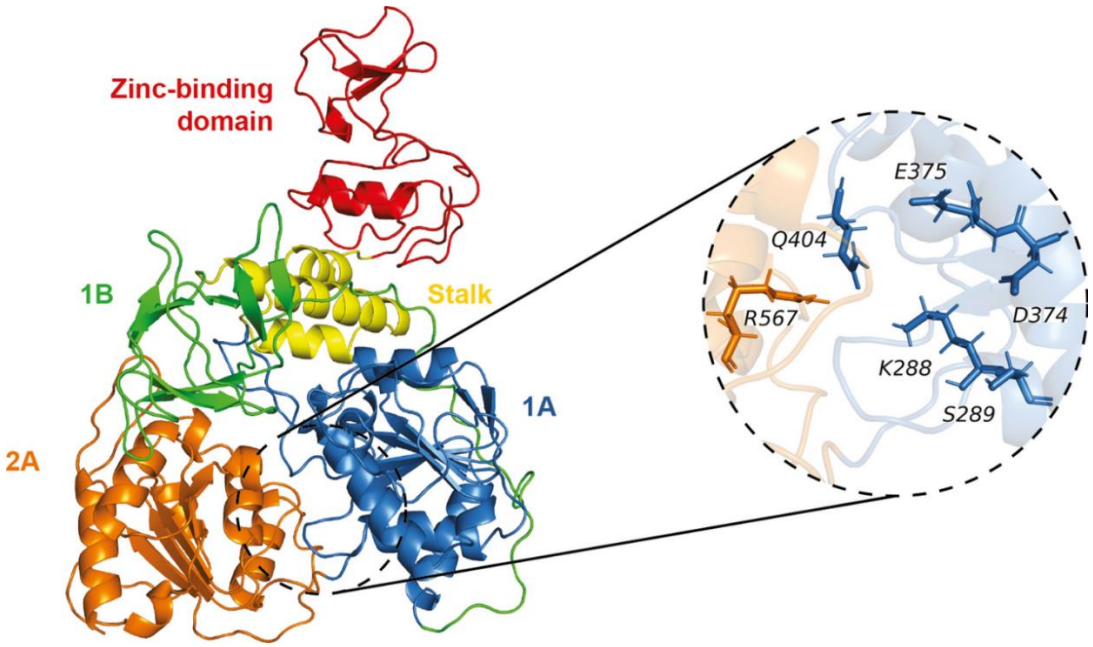

Figure 10 The cartoon representation of nsp13 helicase. Zinc-binding domain (red), Stalk domain (yellow), 1B domain (green), RecA1 domain (blue) and RecA2 (Orange)[40].

Nsp13 helicase is a triangular pyramid-shaped protein that contains five relevant domains namely the N-terminal zinc-binding domain (ZBD), the 1B domain, the Stalk domain, and two C-terminal RecA-like ATPase domains (RecA1 and RecA2) (**Figure 10**)[38]. All domains are required for the enzymatic function of nsp13 helicase[40]. As current day research continues to understand the structure and functions of nsp13 helicase, much remains to be elucidated.

The helicase binds the 5' cap end of a single-stranded RNA template and directs it to the active site of RdRp during elongation[4]. Prior to viral replication and transcription, nsp13 is responsible for the nucleotide triphosphate (NTP)-dependent unwinding of 5' to 3' double-stranded RNA (dsRNA) into single-stranded RNA[4]. Nsp13 also regulates transcriptional elongation by facilitating backtracking of the RdRp[4]. Additionally, nsp13 helicase disrupts protein-RNA interactions, antagonizes IFN, and facilitates mRNA capping via RNA 5' triphosphatase activity[39].

Nsp13 are highly conserved among coronaviruses with >99% sequence similarity between SARS-CoV-1 and SARS-CoV-2[39]. The five aforementioned binding domains, essential for nsp13 its enzymatic activity, are also highly conserved amongst coronaviruses[41]. Point mutations in the 1B domain and the stalk domain reduce helicase activity in SARS-CoV-2 variants[39]. Other mutations such as the nsp13^{R392C} found in the Omicron variant of SARS-CoV-2, show similar helicase activity as the unmutated wild-type nsp13[38].

Antivirals

Current SARS-CoV-2 treatment

COVID-19 does not have a targeted therapeutic intervention. Preventative measures are taken for mild cases including the COVID-19 vaccines, physical distancing, and face masks. For severe cases, treatment efficacy is the highest with early administration[6]. With ARDS, the gas exchange in the lung is reduced while pro-inflammatory immune responses are widespread[6]. To aid effective respiration, oxygen therapy, and ventilation procedures are applied[6]. Corticosteroids such as dexamethasone and budesonide reduce inflammation both systemically and in the lung, reducing mortality significantly[6]. Complementary treatments include the use of Janus kinase inhibitors, cytokine antagonists, and anticoagulants[36].

As of June 2023, 712 compounds with anti-SARS-CoV-2 activity either in preclinical or clinical studies have been reported[36]. The majority of which are small molecules, followed by monoclonal antibodies and peptide inhibitors[36].

Nsp5 (M^{pro}) and Nsp12 RdRp are targets at the forefront of small molecule inhibitor design, due to their well-established binding pockets which share no homology with human proteins[36]. Examples include nirmatrelvir and ensitrelvir as nsp5 inhibitors and remdesivir and molnupiravir as nsp12 inhibitors[36,42]. High conservation of binding pockets allows for inhibitor development with high specificity and reduced off-target effects[36].

Monoclonal antibodies (mAbs) have relatively low efficacy compared to the alternatives[36]. Reliance on binding the SARS-CoV-2 spike protein, which is prone to mutate, has reduced the efficacy of mAbs with new variants of the virus[36].

PROTACs are a promising drug modality that has been recently applied for antiviral purposes. Antiviral PROTACs and other antivirals can have two main strategies by which they are designed, a host-targeting approach and a virus-targeting approach.

Host-targeting antivirals

Antiviral drug design can be divided into two strategies, a host-directed therapy (HDT) or a virus-directed therapy (VDT). HDT is an indirect approach to target viruses by modulating one of several host mechanisms that are essential for the virus and non-essential for the host's function[43]. One common HDT strategy is to target host proteins involved in viral replication and survival e.g. small molecule inhibition of a host protease involved in viral entry[43]. Another strategy involved modulating the host's adaptive immune response to the viral infection to reduce cytokine storms, prevent hyper-

inflammation, and reduce tissue damage[44]. HDT is often used as a combination therapy with a more selective virus-directed antiviral to ensure maximal efficacy[44].

HDT minimizes the chance of drug-resistant variants emerging due to the virus reliance on the host cellular mechanisms, these mechanisms are infrequently mimicked or replaced by viral mutations[44]. Additionally, if several virus species exploit the same host mechanisms, the host-directed drug can be applied as a broad-spectrum antiviral[44]. Broad-spectrum antivirals are of high importance in combatting novel epidemics with relatively unknown viral species, [45].

Targeting prevalent host processes with HDT does however have an increased risk of producing on-target side effects and toxicity compared to VDT[44]. Exogenous, recombinant IFN therapy for the treatment of hepatitis C is an example of HDT that modulates host immune responses to the virus but produces inflammation as a side effect[44]. The indirect targeting of viruses using HDT also reduces the efficacy of antivirals[46].

Virus-targeting antivirals

VDT is the most commonly used antiviral approach that directly targets the virus' enzymes, structural proteins, or its genome[46]. Direct targeting of VDT improves the specificity and efficacy of these antivirals compared to HDT-based antivirals[46]. A viral target that is desirable requires multiple properties to be effective for VDT[46].

First, the inhibition or degradation of the target must cause a loss of an essential function of the virus, thus reducing virulence[46]. This involves targeting proteins that are essential for viral replication, viral entry into the host, or proteins involved in immune modulation. Common viral targets are proteases, structural proteins and polymerases, due to their vital role in replication of viral genomes[47].

Second, targets with high amount of sequence conservation across genetic variants of the virus are desirable. High sequence conservation indicates that the target is indispensable for the viral survival, and any alterations to the sequence will impair viral survival[41]. Compared to other protein targets, highly conserved proteins are less likely to develop drug-resistance, as this would compromise the function of the protein[41]. Antivirals targeting evolutionarily conserved proteins also allows for broad-spectrum application for variants of the virus of interest[41].

Third, viral targets must share minimal homology with similar human analogues. Non-homology ensures the antiviral specifically targets viral proteins and not human proteins, thus reducing side-effects[41].

An additional consideration for a virus-directed approach is that the target should be comprehensively characterized in terms of its function, structure, PPIs, mutant variants,

and relevant binding pockets[48]. Structural knowledge informs the rational design of small molecule inhibitors for enzymatic active sites[48]. Validated small molecule inhibitors with high binding affinity and specificity can be incorporated into antiviral PROTAC design.

Antiviral PROTACs

Host-directed antiviral case study

Hahn et al. developed a CRBN-based cyclin-dependent kinase 9 (CDK9) degrading PROTAC in 2021, as a human cytomegalovirus (HCMV) antiviral[49].

The PROTAC design is an example of HDT due to the target being a host protein that is hijacked by HCMV for viral replication[49]. Normally CDKs play important roles in the control of cell division and modulate transcription in response to several extra- and intracellular cues[50]. CDK9 binds to cyclin T1 to produce the positive transcription elongation factor (P-TEFb) complex[50]. The complex phosphorylates the RPB1 subunit of RNA polymerase II, increasing transcriptional elongation of host and viral mRNA[50]. CDK9 is thus a promising host protein to inhibit or degrade. Targeting host proteins reduces the chance of HCMV mutant variants emerging[44]. Other viruses that exploit CDK9 for replication also have the potential to be targeted by a host-directed PROTAC, allowing broad-spectrum antiviral application. However, targeting CDK9 which is ubiquitously expressed in host cells increases the chance of toxicity and side effects.

The research mainly describes the evaluation of the PROTAC, THAL-SNS032, its antiviral activity various viruses [49]. THAL-SNS032 is comprised of a CRBN recruiting thalidomide anchor connected with a secondary amine to a PEG linker (**Figure 11**). The SNS032 warhead is a potent, selective CDK9/7 inhibitor that is connected to the linker with an amide.

The antiviral capabilities of THAL-SNS032 were evaluated and showed potent in vitro antiviral activity in HCMV, with an EC_{50} range of 0.03 to 3.07 μM depending on the cell type. HCMV strains were tested in HFF, TEV-1, and MRC-5 cell lines. Compared to the small molecule inhibitor SNS032 alone, the PROTAC had a 3.7-fold greater antiviral activity in the same cell type. Cytotoxicity evaluation of the PROTAC resulted in a selectivity index of 5-13, indicating theoretically that the PROTAC is a potent antiviral with low toxicity.

THAL-SNS032 did not show an increase in antiviral efficiency compared to SNS032 in murine CMV models, SARS-CoV-2 infected Caco-2 cells, Zika virus-infected Vero cells, and human adenovirus type 2 (HAdV-2) in HFF cells. THAL-SNS032 showed promising antiviral capability in HCMV and its variants while showing limited application in other viruses. Reduced antiviral activity could be due to differences in CRBN expression in the tested cell lines, or because the viruses do not rely on CDK9 for viral replication.

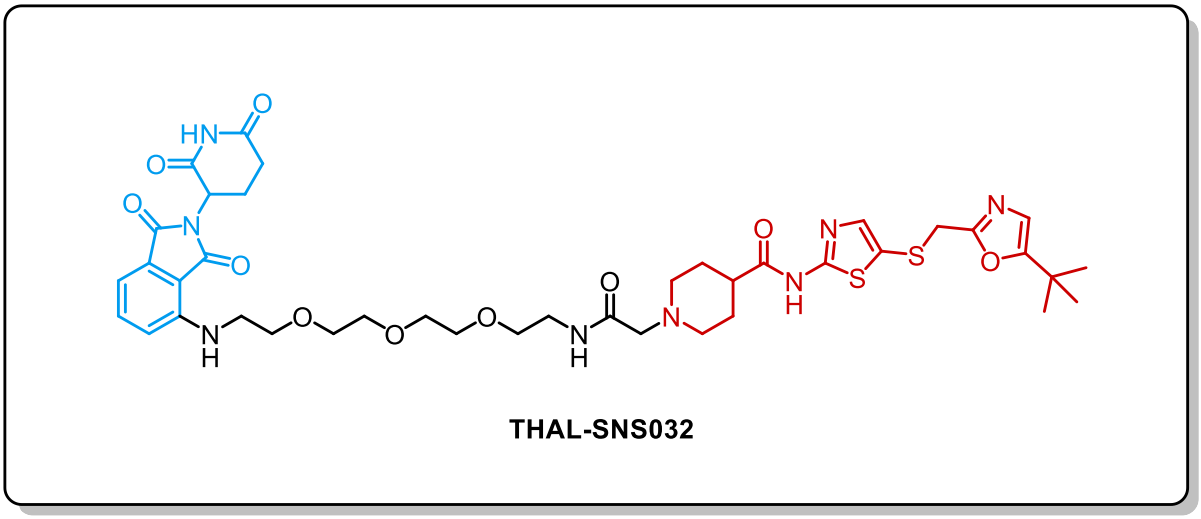

Figure 11 The chemical structure THAL-SNS032. It is comprised of a thalidomide anchor (blue), a PEG linker (black), and an SNS032 warhead (red).

Virus-targeting antiviral case study

Alugubelli et al. designed a first-in-class PROTAC degrader of SARS-CoV-2 its main protease (M^{pro} or Nsp5) in 2024[51]. The study encompassed the design, synthesis, and in vitro testing of degradation capabilities of ten bifunctional M^{pro} PROTAC degraders, MPD1-MPD10.

Targeting M^{pro} using PROTACs constitutes a virus-targeting antiviral approach. M^{pro} possesses several desirable properties for VDT. First, M^{pro} is a protease that is essential for SARS-CoV-2 RNA replication and survival, its degradation would lead to essential loss of function for the virus. Second, M^{pro} is a highly conserved enzyme amongst SARS-Cov-2 variants, reducing the generation of mutant variants and allowing for broader antiviral applications[41]. Third, M^{pro} is non-homologous to human proteases, reducing off-target effects and increasing selectivity[41].

Previous research from this group developed the potent M^{pro} binding reversible covalent inhibitors, MPI8 and MPI29, which were incorporated into the PROTAC as a warhead[51]. MPI8 specifically, showed high inhibition potency and a high binding affinity to the P1- and P2-active sites of M^{pro}. MPD1-MPD6 were based on MPI8 and MPD7-10 were based on MPI29.

All ten degraders recruited CRBN E3 ligase with an anchor consisting of the established ligands thalidomide-4-OH and pomalidomide. The warhead and anchor were connected with a variety of linker lengths and compositions, with functional groups including PEG, alkyl, tertiary amides, and tertiary amines.

Inhibition of SARS-CoV-2 M^{pro} by all ten compounds was determined via fluorescence polarization assay. All ten compounds displayed sub-micromolar IC_{50} values, demonstrating the effective inhibition of M^{pro}, regardless of E3 ligands or linker design.

A cellular degradation assay was then performed using genetically modified 293T cells which express M^{pro} attached to an eGFP fusion protein. Cell lines were treated with all MPD1-10 after which the half-maximal degradation concentration (DC^{50}) was determined by Western blot analysis. Out of all compounds, MPD1-MPD3 showed the most efficient degradation profiles with DC^{50} values of 419, 296, and 431 nM respectively. MPD1-3 was chosen to test further and was then screened for cytotoxicity in 293T cells using an MTT assay. The resulting half-maximal cytotoxic concentration (CC_{50}) for MPD1-MPD3 were 25, 120, and 21 µM respectively. Due to the low cytotoxicity combined with quick and efficient M^{pro} degradation, MPD2 was chosen for further testing.

MPD2 consists of a thalidomide CRBN E3 ligase ligand, a PEG/alkyl linker, and the reversible covalent M^{pro} inhibitor warhead MPI8 (**Figure 12**). Mechanistic control studies for MPD2 were conducted to validate the event-driven protein degradation mechanism, confirming its dependence on the CRBN E3 ligase, the presence of M^{pro}, and the UPS.

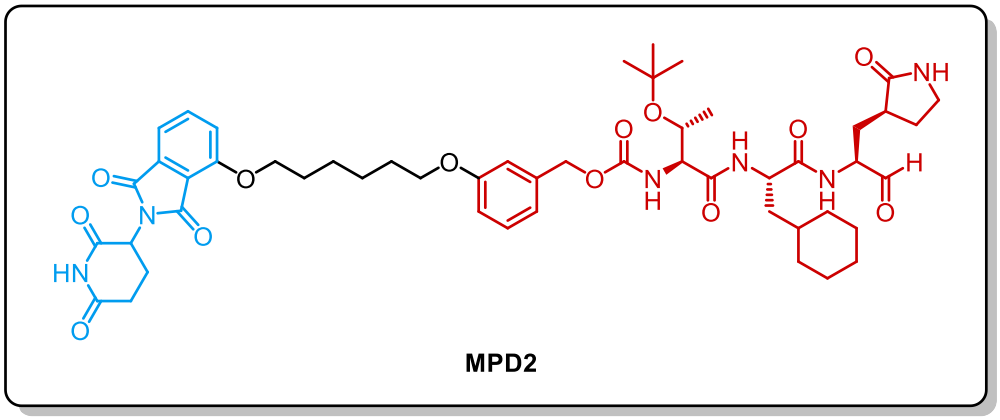

Figure 12 The chemical structure of M^{pro} degrading PROTAC MPD2. It is comprised of a thalidomide anchor (blue), an alkyl linker (black), and an MPI8 warhead (red).

The antiviral efficacy of MPD2 was tested in A549-hACE2 cells that were infected with SARS-CoV-2, including the variants WA.1 BA.1 and XBB.1.5. At 2.5 µM, MPD2 reduced viral replication by 90% in A549-hACE2 cells of all variants. Additionally, A549-hACE2 cells with an NSP5 E166A mutation, a strain that is nirmatrelvir resistant, were treated with MPD2. MPD2 showed 5-fold inhibition of viral RNA levels as compared to the wild-type variant.

In conclusion, MPD2 is a promising first-in-class M^{pro} degrading, antiviral PROTAC with an efficient degradation profile. This CRBN-based PROTAC has the potential to treat wild-type SARS-CoV-2 strains as well as mutated variants.

Theoretical nsp13 helicase degrading PROTAC design

This work presents a theoretical nsp13 degrading PROTAC for SARS-CoV-2, including a CRBN-based anchor and an SSYA10-001 inhibitor-based warhead. Nsp13 helicase is a promising experimental viral target for antiviral drug design. As of December 2024, there are no PROTAC degraders of nsp13 helicase reported in the literature. Mostly due to incomplete characterization of nsp13 its functions, interactions with other proteins, and lack of available small molecule inhibitors. Targeting nsp13 helicase is a virus-targeting strategy.

Virus-targeting antiviral approach

Nsp13 has multiple characteristics that make it an exciting prospect for PROTAC-based VDT. First, nsp13 is a multi-functional enzyme necessary for viral replication as it unwinds, and rearranges dsRNA before transcription[4]. Nsp13 degradation would negate viral transcription and replication sufficiently to produce an antiviral effect. Peripheral functions of nsp13 would also be reduced by TPD, such as mRNA capping and host immune response modulation[4]. IFN antagonization by nsp13 would be downregulated by nsp13 proteolysis resulting in an improved immune response by the host[4].

Second, nsp13 is highly conserved across several coronaviruses and their variants[52]. An effective nsp13 degrading PROTAC can therefore be broadly applied to newly emerging coronaviruses, aiding the prevention of novel epidemics. Furthermore, nsp13 its high sequence conservation reduces the chance of mutation-induced drug resistance. It is important to note that SARS-CoV-2 variants develop point mutations in new variants as is the case with Omicron nsp13^{R392C} [38].

Third, nsp13 is mostly non-homologous to human helicases, with some important distinctions[53]. Coronavirus helicases contain several motifs similar to essential host factors. A promiscuous PROTAC could therefore degrade host proteins which could lead to severe effects seen in Bloom's and Werner's syndrome[53]. These adverse effects can be mitigated by ensuring high specificity of the PROTAC and targeting nsp13 active sites which are comparatively less homologous to human helicases[53].

PROTAC design

For the design of the nsp13 degrading PROTAC, the MedChem toolbox for CRBN-based PROTACs by Steinebach et al. was used for the CRBN ligand[26]. The PROTAC incorporates the CRBN E3 ligase recruiting ligand 4-aminothalidomide (pomalidomide) (**Figure 13**). Targeting the CRL4^{CRBN} complex has the benefit of being ubiquitously expressed, ensuring sub-cellular co-localization of the E3 ligase and the target POI[12]. CRBN-ligands such as pomalidomide are well-characterized in terms of degradation profile, potency, and specificity[18]. Furthermore, the ligand is small, relatively cheap, accessible, and has

pharmacokinetically favorable physicochemical properties that aid the reported poor ADME of PROTACs[18]. CRBN-based PROTACs also have comparatively efficient degradation profiles compared with alternative E3 ligands.

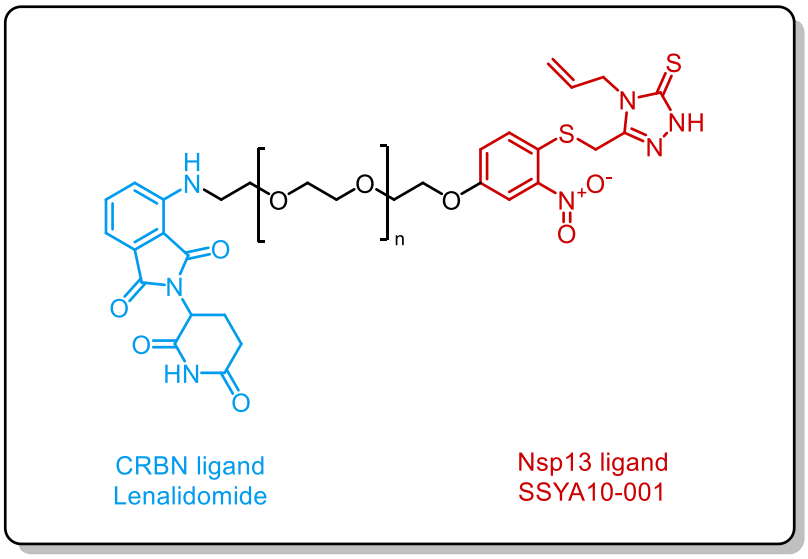

Figure 13 The chemical structure of the proposed nsp13 degrading PROTAC. It is comprised of a lenalidomide anchor (blue), a PEG linker (black), and an SSYA10-001 warhead (red).

The toolbox has informed the initial attachment site of the linker to the two ligands in **Fig. 3**[26]. As the rational design of linkers is complex due to unpredictable linker SAR, the proposed structure is subject to change in terms of linker attachment site, linker length, and linker functional group composition. The toolbox of Steinebach et al. can be used to systematically modify the initial PROTAC linker design to attain the desired physicochemical properties[25,26]. The aromatic amino group of the pomalidomide is attached to the linker using aliphatic linker carbons, similar to that of ARV-825[26]. The bulk of the linker consists of PEG, which alternatively be substituted by other commonly used linker moieties including alkyl, alkyne, glycol, triazole, piperazine, or piperidine motifs[54].

The opposing end of the linker contains the nsp13 inhibitor SSYA10-001, which is connected with an ester. SSYA010-001 (3- [(2-nitrophenyl)sulfanylmethyl]−4-prop-2-enyl-1H-1,2,4-triazole-5-thione) was first reported in 2012, by Adedeji et al. in an extensive FRET screening of a compound library of 3000 potential SARS-CoV-1 nsp13 helicase inhibitors[55]. The study found SSYA010-001 to be a specific, non-competitive inhibitor of nsp13 helicase activity for SARS-CoV-2, with an IC_{50} of 5.7 µM[55]. Additionally, no significant cytotoxicity was observed even at the highest tested concentration 500 µM[56]. Since the initial studies in 2012 and 2014, the compound has become increasingly more established concerning its inhibitory potency and limitations.

SSYA010-001 is a 1,2,4-triazole derivative that targets highly conserved regions in the RecA1 domain of nsp13[38]. Due to the high conservation of the binding pocket, the inhibitor functions as a pan-inhibitor of coronaviruses[38]. Although the specific mechanism of action is unknown, it is postulated that the inhibitor inhibits unwinding

activity independent of ATP- or nucleic acid binding pockets[38]. The inhibitor binds an unspecified motif in the RecA1 domain of nsp13[38]. This region is highly conserved but infrequent mutations such as Y277A and K508A produce SSYA010-001 drug-resistance[56].

In January of 2024, Inniss et al. evaluated SSYA010-001 and its inhibitory potency for the nsp13 of SARS-CoV-2[38]. Using a FRET-based assay the inhibitor displayed inhibition of wild-type nsp13 with a sub-micromolar IC_{50} of 0.64 ± 0.03 μM. Similar sub-micromolar IC_{50} values have been reported by Nizi et al. (0.046 ± 0.015 μM) and Corona et al. (0.05 ± 0.02 μM)[57,58]. The compound was also tested on nsp13 containing a point mutation prevalent in the Omicron variant, namely $nsp13^{R292C}$ [38]. SSYA010-001 showed similar inhibitory potency (IC_{50} = 0.61 ± 0.22 μM) for the mutated variant as compared to the wild-type nsp13, indicating its potential in combatting variants of SARS-CoV-2.

The incorporation of SSYA010-001 is experimental due to the many unelucidated properties and interactions with nsp13. The interactions of nsp13 with other nsps and host proteins must be further researched to determine the efficacy and binding affinity of SSYA010-001. In particular, nsp12 has been indicated to increase the helicase activity of nsp13, which was not evaluated in the research of Inniss et al.[38]. Furthermore, the specific binding site needs to be elucidated to optimize the inhibitor and to establish any structural homology with human helicase motifs.

Structural modifications can be made to SSYA010-001 to improve physicochemical properties and further enhance binding potency. Spratt et al. proposed several in silico-derived derivatives of SSYA010-001 which showed similar physicochemical properties (**Figure 14**)[59]. These derivatives remove the problematic aromatic nitro group, which is prone to be metabolized by CYP-P450 to carcinogenic nitrosamine groups[60]. The derivatives should be tested for nsp13 inhibition potency and affinity as an alternative to SSYA010-001.

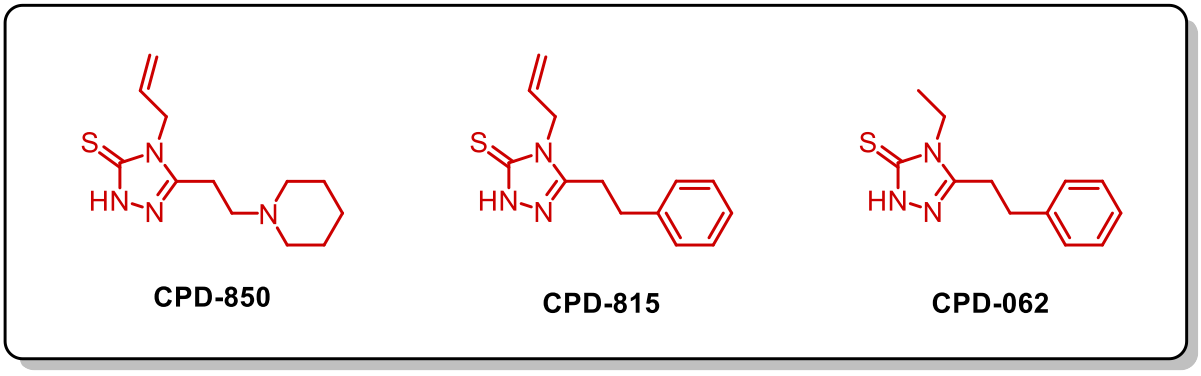

Figure 14 The chemical structures of SSYA010-001 warhead derivatives CPD-850, CPD-815, and CPD-062 by Spratt et al.[59].

Once the optimal warhead has been discerned, it can be incorporated into the PROTAC. The 1,2,4-triazole moiety is responsible for binding the nsp13 active site, thus the linker is attached to the side of the inhibitor opposing the triazole moiety.

Discussion & Conclusion

This essay proposes a theoretical nsp13 degrading PROTAC for SARS-CoV-2. The PROTAC consists of a CRBN recruiting pomalidomide anchor, a PEG linker, and an nsp13 inhibitor SSYA010-001 inhibitor warhead. The design of the PROTAC is purely theoretical and based on the limited available literature regarding nsp13 and its inhibitors. Experimental data from in vitro and in vivo studies is required for the validation of all three PROTAC moieties, ternary complex formation, protein degradation profiles, and toxicity.

The emergence of the SARS-CoV-2 epidemic that started in late 2019 has put exceptional strain on the global medical and economic sectors. Over 7 million reported deaths have been caused by SARS-CoV-2-induced COVID-19 with over 100-fold more people being infected. The medical need for antivirals that combat coronaviruses and their variants is of great importance to curb upcoming variants and epidemics. In particular, pan-coronavirus antivirals that treat drug-resistant variants of SARS-CoV-2 are of crucial importance. Antiviral PROTACs have gained traction as they are a potent drug modality effective in treating small molecule drug-resistant diseases.

While the SSYA010-001 warhead has promising nsp13 inhibitory functionality, further information is needed about nsp13 its binding pockets, and mechanism of action to reliably optimize the inhibitor for PROTAC incorporation. The limitations of SSYA010-001 are mainly due to its uncharacterized binding motif and mechanism of action. It is unclear whether the motif is homologous to human helicases, raising potential toxicity concerns. Moreover, interactions with nsp12 or host factors could negatively influence the binding affinity of the warhead, which needs to be confirmed experimentally.

Additionally, linker composition and attachment sites should be systematically tested to obtain the desired nsp13 degradation profile, flexibility, ternary complex formation, and pharmacokinetic parameters. The CRBN ligand pomalidomide is a reliable, potent, selective, and well-established ligand. If pomalidomide does not produce the expected TPD, the linker composition or attachment site can be altered or an alternative CRBN recruiting ligand can be employed.

Once the warhead and the anchor have been validated in terms of their binding affinity and potency to their respective targets, the PROTAC can be evaluated. Antiviral PROTAC validation includes experimental determination of the ternary complex stability as this is one of the main contributors to effective TPD. Biochemical assays such as SPR and ITC can quantify and confirm degradation-competent ternary complex formation in vitro. The PROTAC its selectivity and specificity need to be of a high standard, as degradation of host helicases has severe side effects for patients. If nsp13 degradation profiles are effective and selective, the antiviral activity can be tested in SARS-CoV-2 infected cell lines, and potentially other coronaviruses to see if the PROTAC has pan-coronavirus application.

As the field is on the cutting edge of drug design, strategies for designing antiviral PROTACs are sparse and in need of further research. Antiviral PROTACs show potential to be an exciting new prospect in the treatment of viral infections do to their unique mechanism of action.

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