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Perspective

# Targeted Protein Degradation for Infectious Diseases: from Basic Biology to Drug Discovery

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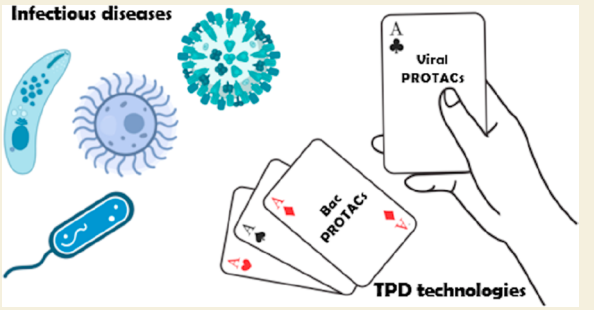
**ABSTRACT:** Targeted protein degradation (TPD) is emerging as one of the most innovative strategies to tackle infectious diseases. Particularly, proteolysis-targeting chimera (PROTAC)-mediated protein degradation may offer several benefits over classical anti-infective small-molecule drugs. Because of their peculiar and catalytic mechanism of action, anti-infective PROTACs might be advantageous in terms of efficacy, toxicity, and selectivity. Importantly, PROTACs may also overcome the emergence of antimicrobial resistance. Furthermore, anti-infective PROTACs might have the potential to (i) modulate "undruggable" targets, (ii) "recycle" inhibitors from classical drug discovery approaches, and (iii) open new scenarios for combination therapies. Here, we try to address these points by discussing selected case studies of antiviral PROTACs and the first-in-class antibacterial PROTACs. Finally, we discuss how the field of PROTAC-mediated TPD might be exploited in parasitic diseases. Since no antiparasitic PROTAC has been reported yet, we also describe the parasite proteasome system. While in its infancy and with many challenges ahead, we hope that PROTAC-mediated protein degradation for infectious diseases may lead to the development of next-generation anti-infective drugs.




**KEYWORDS:** *Targeted protein degradation, ubiquitin proteasome system, PROTACs, infectious diseases, anti-infective drug discovery, pathogens*

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## INTRODUCTION

The term "infectious disease" refers to a pathological process caused by microorganisms such as bacteria, viruses, fungi, parasites, or, in a peculiar case, prions. Although most of these agents are harmless or even beneficial to humans, under certain circumstances, they may cause diseases with different degrees of severity. The history of mankind is punctuated by devastating epidemics generated by such microorganisms, starting from the plague of the Middle Ages, the Spanish Flu during the First World War, HIV/AIDS in the 1980s, up to the, yet undefeated, SARS-CoV-2 pandemic. The latter, which has unfortunately become our daily routine over the past two years, has caused a heavy death toll, not to mention the high, and often unsustainable, economic, social, and psychological costs that it is leaving behind. However, this pandemic has allowed the general public to open their eyes to the devastating and potentially deadly effects of infectious diseases and to the importance of drug discovery research to tackle them. Indeed, antimicrobials are among the most successful drugs in the history of medicine. Although the use of antibiotics can be tracked to over 2000 years ago, the so-called "modern antibiotic era" began in the past century, thanks to the work of Paul Ehrlich and Alexander Fleming and the discovery of Salvarsan, Prontosil, and penicillin.[1] According to a 2014 analysis, 292 new chemical entities (NCEs) have been developed to treat infectious diseases since the Food and Drug Administration (FDA) approval of sulfapyridine in 1939.[2] Interestingly, the number of NCEs peaked during the 1990s and declined rapidly thereafter.[2] Since 2000, only a small number of antibiotics have been approved (<20) and among them only four are characterized by a new mechanism of action (MoA). The reasons behind the decline in antibiotic research and development (R&D) over the past two decades are different. Developing a new antibiotic is extremely difficult, with an estimated failure rate of 95%, and costs of hundreds of millions of U.S. dollars.[3−5] This means that it is no longer cost-effective for the major players in global drug discovery.[6] Furthermore, some microbes (either bacteria, viruses, fungi, or parasites) change over time (the so-called *superbugs*), and

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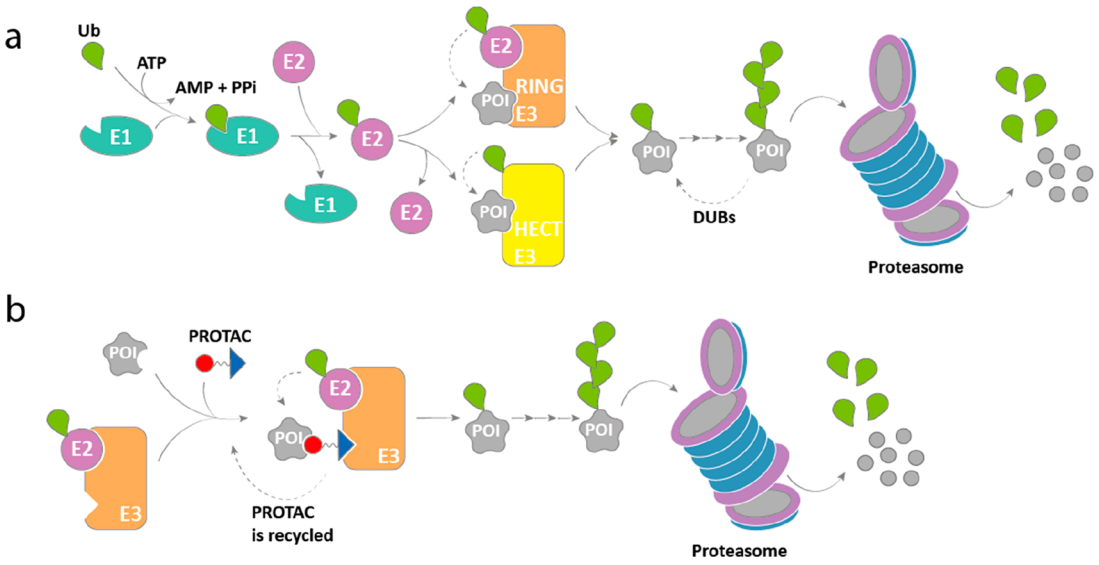


**Figure 1.** Schematic representation of the UPS and PROTAC technology. (a) Scheme of the ubiquitylation cascade leading to the proteasomal degradation of the POI. (b) Schematic representation of PROTAC's MoA.

antimicrobial resistance (AMR) occurs when they no longer respond to medicines commonly used to treat the infections they cause. Broadly speaking, mechanisms of AMR fall into three categories: target modification, drug inactivation, and drug transport.[7] In bacteria, in addition to these canonical mechanisms, formation of biofilms further complicates AMR management (e.g., resistance to ceftazidime in biofilm-growing *Pseudomonas aeruginosa*).[7] In viruses, their ability to replicate quickly makes the selection of resistant strains with altered antiviral targets the main AMR mechanism (e.g., resistance to telaprevir is due to mutations in the NS3−4A protease of the hepatitis C virus).[8] As for antiparasitics, drug resistance is more generally associated with loss-of-function mutations in the transporters involved in drug import[9] (e.g., the pentamidine resistance protein 1 (PRP1) and the aquaglyceroporin 2 (AQP2) are the transporters responsible for resistance to pentamidine and melarsoprol in Trypanosomatids).[10]

Based on the World Health Organization (WHO), AMR is "*one of the top 10 global public health threats facing humanity* and *requires a global, coordinated action plan to address*".[11] New analysis finds out that AMR contributed to the deaths of 4.95 million people only in 2019.[12] AMR is likely to have a higher global burden than HIV or malaria and is inevitably bringing humanity toward the "post-antibiotic" era.[13] Thus, the world desperately needs next-generation antibiotics and antiparasitic molecules, as well as innovative strategies to combat infections caused by superbugs and emerging and reemerging pathogens. In this framework, targeted protein degradation (TPD) performed by the so-called ubiquitin-proteasome system (UPS), is being considered as one of the most feasible pharmacological strategies.

UPS controls many cellular processes such as cell cycle progression and signaling. Ubiquitylation is a reversible post-translational modification, ultimately leading to proteasomal protein degradation (Figure 1a). Deubiquitylating enzymes (DUBs) play a crucial role in the regulation of ubiquitin-controlled processes by reverting the ubiquitylation status of proteins.[14] The conjugation of ubiquitin (Ub) to substrates is a multistep process that requires the sequential activity of three classes of proteins. The first step is carried out by the ubiquitin-activating enzyme E1 (UBA1).[15] The Ub is first adenylated at the C terminus, and then transferred at the catalytic E1 cysteine residue, so that a high energy E1-Ub thioester conjugate is formed. A second Ub molecule dislocates the thioester bound Ub from the E1 active site, inducing its transfer to the catalytic cysteine of the ubiquitin-conjugating enzyme E2.[16,17] Ubiquitin ligase (E3) is responsible for the selection of target proteins and interacts with both the E2-Ub thioester complex and the protein of interest (POI) to which Ub is meant to be transferred.[18] Depending on their MoA, E3 ligases can be classified in two groups: a smaller group, including the HECT and RING-in-between-RING proteins, which form a covalent intermediate with Ub via a trans-thioesterification reaction before transferring it to substrates, and a larger one, containing the majority of RING and U-box proteins, in which the Ub transfer to the substrate lysine occurs directly from E2 to the substrate via an aminolysis reaction. E3 proteins can either act as single proteins or be part of larger multiprotein complexes. By hijacking the UPS, proteolysis-targeting chimeras (PROTACs) have recently received a great deal of attention as a new therapeutic modality based on the modulation of protein levels.

PROTACs are chimeric small molecules[19] consisting of a ligand binding an E3 ubiquitin ligase[20] and one recognizing a POI, properly connected via a suitable linker.[21] Once the E3-PROTAC-POI ternary complex is formed, an E2 enzyme is recruited and the POI is polyubiquitylated (Figure 1b). This chemically induced proximity between the POI and the E3 ubiquitin ligase elicits ectopic polyubiquitination and subsequent proteasomal degradation.

In the past 20 years, TPD has emerged as a relevant and versatile tool for advancing drug discovery both in industry and in academia.[22] PROTACs harness the cellular degradation system to selectively degrade pathological proteins in a catalytic event-driven mechanism, unlike the occupancy-driven approach of conventional drugs.[23] This eliminates the need of an active site and allows pursuing targets that were previously considered "undruggable".[23,24] Already applied to oncology, immune disorders, neurodegenerative, fatty liver, and cardiovascular diseases, new applications for PROTAC-mediated


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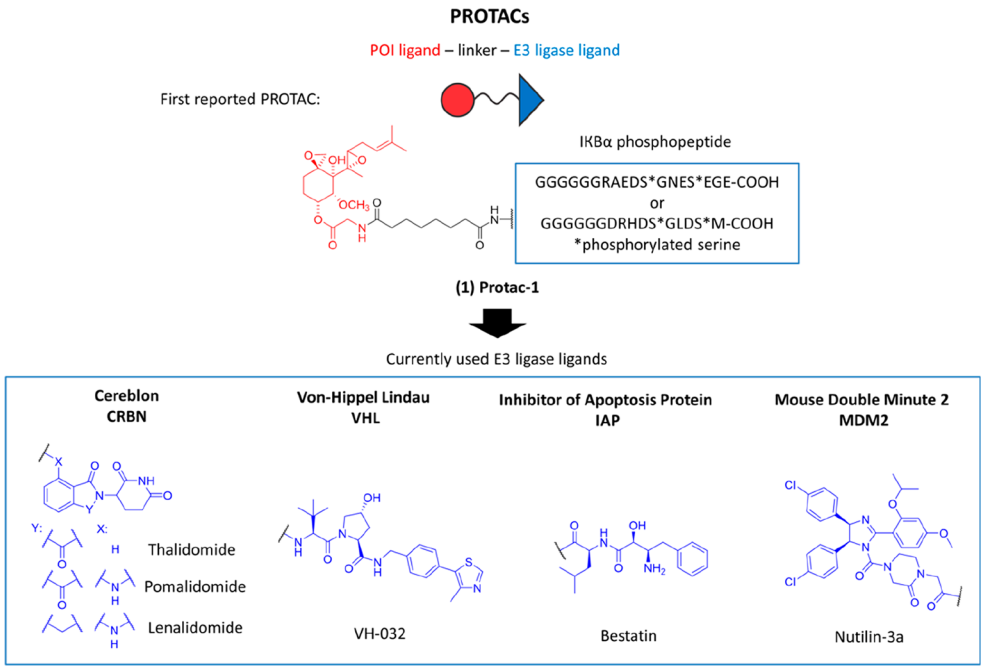


**Figure 2.** First reported Protac-1 (**1**) and the most common E3 ligase ligands.

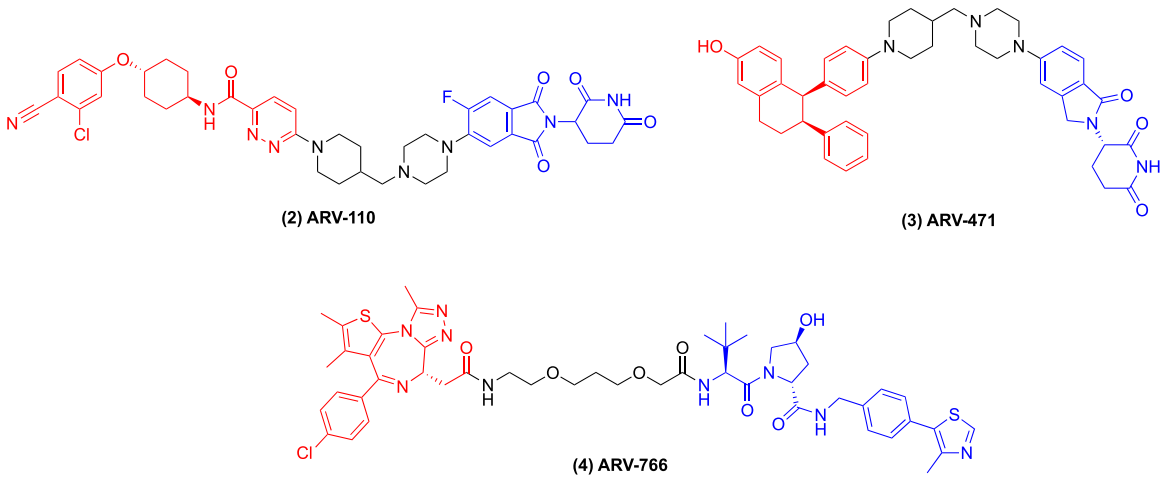


**Figure 3.** Current clinical candidates **2**–**4** developed by ARVINAS biopharmaceutical company.

TPD are emerging every day.[25] It has proven to cover different classes of protein targets, making it particularly attractive for extension into other diseases, including infectious diseases.[26,27]

Remarkably, PROTAC technology has been successfully implemented for preclinical studies for viral diseases[28] and, more recently also for bacterial diseases (BacPROTACs).[29] This represents an exciting opportunity for expanding its scope to other pathogens, for a full exploitation in infectious diseases drug discovery.[26,27] Definitely, this approach needs further study to untap its great potential in relation to (i) the advantage of its catalytic MoA, (ii) the possibility to overcome drug resistance, (iii) the selection of anti-infective targets classified as "undruggable" by the classical occupancy-driven approach, (iv) the "recycling" of inhibitors coming from unsuccessful drug discovery programs, and (v) the chance for combination therapies.

In this Perspective, we will try to address these points by discussing selected case studies of PROTACs developed in the infectious disease area.

## PROTACS: PAST, PRESENT, AND FUTURE

Since the first exploitation of the protein-degradation machinery for the selective degradation of a POI in 2001,[30] the PROTAC approach has evolved quite a bit.[23] In the first application, a chimeric molecule called Protac-1 (**1**, Figure 2) promoted the degradation of the methionine aminopeptidase-2 thanks to the recruitment of the Ub ligase, Skp1-Cullin-F-box (SCF) complex.[30] Despite the promise of this approach, **1** showed limited cell penetration due to the IKB$\alpha$ phosphopeptide. This pointed out the need for finding new E3 ligase ligands with an improved pharmacokinetic (PK) profile. This need was met with the discovery of small-molecule E3 ligase ligands, e.g., Cereblon (CRBN) binding molecules which encompass the immunomodulatory drugs (IMiDs) thalidomide, pomalidomide, and lenalidomide (Figure 2).[31,32] Currently, the most used E3 ligase binders for PROTAC design include also Nutlin-3a for the Mouse Double Minute 2 (MDM2), bestatin for the Inhibitor of Apoptosis Protein


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(IAP), and VH-032 for the Von Hippel−Lindau (VHL;[21] Figure 2).

Intuitively, PROTAC technology is guided by the formation of a ternary complex between the PROTAC, the POI, and the E3 ligase, which promotes protein degradation by the UPS in eukaryotic cells.[19] Thus, understanding the interactions that drive ternary complex formation is an essential factor for advancing PROTAC technology. In this respect, new *in silico* approaches represent promising tools.[33] In addition to this, novel strategies aimed at improving and optimizing PROTAC features, including cell permeability, selectivity, PK profile, in vivo efficacy, and safety, are being developed to further enhance the effectiveness of TPD.[34]

In any case, PROTACs have already proven to be a promising therapeutic modality. The biopharmaceutical company ARVINAS has three candidates in clinical trials for the treatment of prostate and breast cancers (ARV-110 (**2**),[35] ARV-471 (**3**),[36] and ARV-766 (**4**);[37] Figure 3), demonstrating how this approach is quickly progressing to the clinics.

As a future perspective, it should be remarked that PROTAC technology is not restricted to a TPD outcome. A study developed in 2022 reports an innovative application for the generation of live-attenuated vaccines.[38] Preliminary results in mouse and ferret models showed how engineered influenza A viruses bearing a VHL recognition sequence could selectively degrade the modified viral proteins, allowing the construction of an attenuated virus as a next-generation vaccine termed PROTAC virus.[38]

PROTACs, although the first, are not the only type of protein degradation technology that exists today. The field is rapidly expanding, and similar modalities include (among others) AUTACs (autophagy-targeting chimeras) and LYTACs (lysosome-targeting chimeras), i.e., degraders exploiting autophagy and endolysosomal pathways, respectively.[39] Furthermore, a new technology that targets RNAs for degradation (and not a POI) is based on RIBOTACs (ribonuclease-targeting chimeras). RIBOTACs have been recently developed to effectively degrade SARS-CoV-2 RNA.[40] Another proximity-based modality involves DUBTACs (deubiquitinase-targeting chimeras). They, differently from PROTACs, induce ternary complex formation with a deubiquitinating enzyme, driving deubiquitylation and stabilizing the POI.[41]

## POTENTIAL ADVANTAGES OF PROTACS IN INFECTIOUS DISEASES

PROTACs have reached clinical trials in oncology, showing several advantages in comparison to classical approaches.[23] In this regard, what we have learned from oncology applications might be an opportunity to streamline TPD application in the anti-infective field. Similar to the oncology area, the main limitations of currently used anti-infective treatments might include high toxicity and low efficacy, mainly due to drug resistance.[42] This last point is especially alarming, considering the rapid global spread of AMR, as declared by the WHO.[11] Against these drawbacks, PROTAC-mediated TPD may provide benefits over classical small-molecule-mediated inhibition.

**Toxicity:** Some antimicrobial drugs may exhibit high toxicity. Long-term and high-dosage treatment could lead to toxic side effects. Some of the best known examples include chloramphenicol and anemia, amphotericin B and hypokalemia, and aminoglycosides and eighth-nerve toxicity.[43] The lower dose of a PROTAC could in principle guarantee a better safety profile. Due to the catalytic MoA, PROTACs can act within a lower concentration window if compared to traditional drugs, which require a higher drug to POI stoichiometry for an efficient inhibition of the protein function.[23]

**Efficacy:** Antimicrobial drugs may suffer from low efficacy. One of the reasons is that drugs developed so far are directed toward a limited number of validated targets (e.g., inhibition of synthesis of bacterial cell wall, cell membrane alterations, inhibition of protein synthesis and replication). Furthermore, some of them are repurposed (e.g., the antitumor agent eflornithine approved for Human African Trypanosomiasis and the anticancer zidovudine approved for human immunodeficiency virus type 1).[44] This means that they were not even rationally designed to effectively target that POI. Generally, to obtain an effective inhibition over an extended time, exposure to small-molecule inhibitors at sustained and saturating concentrations is required.[45] PROTACs only involve a transient binding event to form a productive ternary complex, which leads to POI degradation. Thus, small-molecule-mediated TPD could offer benefits, i.e., reduced drug exposure and time required to suppress signaling. Moreover, the effect can be prolonged as it depends on the resynthesis rate of the POI. Finally, PROTACs may in principle harness both the host and the pathogen protein degradation machinery, resulting in a more effective treatment.[26]

**Selectivity:** Antimicrobial drugs may lack selectivity. This is especially the case when the target protein possesses a human homologue. By contrast, PROTACs exemplify the concept of "gaining selectivity". In a pioneering work, it was demonstrated that incorporating a promiscuous ligand into a PROTAC provides selective degraders.[46] The basis of PROTAC selectivity arose from cooperativity between the E3 ligase and the POI, which ultimately drove PROTAC selectivity and potency.[46] However, the engaged E3 ligase affected the selectivity of degradation, as VHL-recruiting PROTACs were more selective than CRBN-PROTACs.[46] PROTACs could help to gain selectivity toward pathogen-specific isoforms vs human homologues when starting from an unselective ligand. More importantly, harnessing pathogen-specific protein degradation machinery could boost selectivity. Selectivity might also be improved by incorporating into the PROTAC's structure pathogen-specific targeting moieties (e.g., peptides, antibodies). A selective degradation of POIs in cancer cells versus noncancerous normal cells has been demonstrated for the recently discovered Folate-caged PROTACs.[47]

**Resistance:** The main issue faced by anti-infectives is AMR. As discussed above, AMR arises from pathogen drug-resistant mechanisms such as point mutation, compensatory overexpression, or bypassing the target in the signaling cascade.[48] PROTACs have been successfully developed to address resistance issues in several malignant tumors with different resistance mechanisms.[49] Thanks to PROTAC catalytic MoA, it is unlikely that the decreased binding affinity toward the POI due to a point mutation may impact the formation of the ternary complex and PROTAC's degradation activity. Because of protein level modulation rather than inhibition, PROTACs are also particularly suitable in resistance cases caused by overexpression and scaffolding functions of the POI.[49] Indeed, PROTACs might produce a more complete and long-term inactivation of downstream signaling and avoid the compensatory feedback mechanisms via alternative pathways. However, it should be emphasized that some resistance mechanisms to


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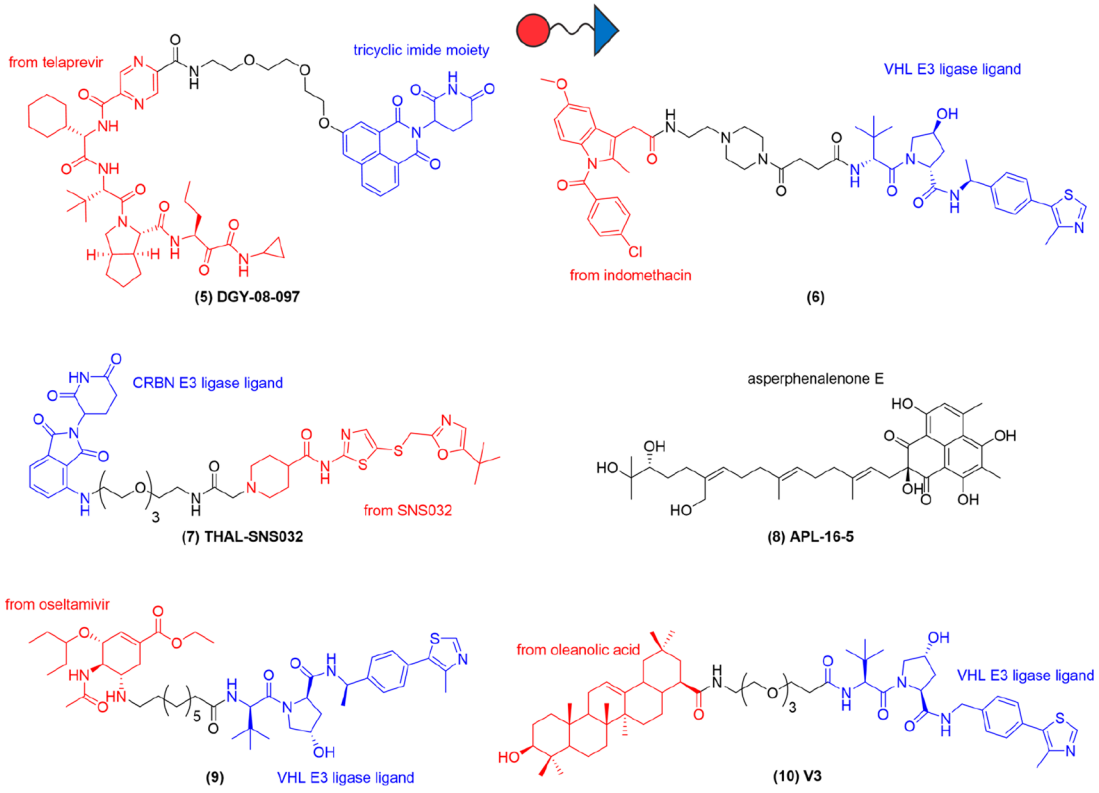


**Figure 4.** Antiviral PROTACs **5**−**10**.

PROTACs have been already identified[50,51] (i.e., genomic alteration in the E3 ligase core), and further studies are required to fully understand this phenomenon. In addition, the recent discovery of "allosteric" degraders has provided a potential strategy to overcome drug-resistant mutations that have emerged within the ATP binding site of oncogenic proteins.[52,53] This is a step forward to further expanding the chemical space available for both anti-infective and anticancer drug development. Notably, in the search for new anti-infectives, the development of allosteric enzyme inhibitors is already considered a welcome alternative to classical inhibitors targeting the orthosteric binding site.[54]

Learning from what has been already achieved, anti-infective PROTACs might also have the potential to (i) cover "undruggable" targets, (ii) "recycle" inhibitors from classical approaches, and (iii) open new scenarios for combination therapies.

### i. Cover Undruggable Targets

It is widely accepted that the affinity of the ligand rather than its pharmacological activity (agonist, antagonist, or inhibitor) is the starting point for PROTAC design. This aspect has represented a good opportunity for modulating those classes of proteins that lack druggable binding sites (e.g., transcription factors[55] and scaffolding proteins[56]) or are not effectively modulated by small molecules. PROTACs have been indeed confirmed to modulate "undruggable" targets in oncology.[57] In anti-infective drug discovery campaigns, several target-based approaches have been abandoned in favor of phenotypic ones, currently widely exploited to identify new anti-infective hits.[58] Featureless and broad binding sites (e.g., viral N-glycans and glycan-binding proteins[59] and trypanothione reductase[60]) and protein−protein interactions that are difficult to disrupt (e.g., Ebola viral protein 35/double-stranded RNA complex[61] and bacterial flagellin/host protein complex[62]) have hampered anti-infective target-based drug discovery endeavors. In this respect, PROTACs could help to broaden the possibilities to modulate and degrade those anti-infective targets deemed "undruggable".

### ii. "Recycle" Inhibitors from Traditional Drug Discovery Campaigns

Anti-infective drug discovery campaigns have produced tons of ligands, which were discarded mostly due to a poor PD−PK profile and/or toxicity issues.[63] The application of the PROTAC strategy might instead give new life to such discarded compounds. As for the PD profile, even a low-affinity ligand could provide effective PROTACs, which opens up new scenarios for weak binders. Regarding the PK, recent advances in the design of orally bioavailable PROTAC degraders, together with the evidence that they can enter the brain, widen the applicability of PROTACs.[64] Concerning toxicity, as discussed above, a lower dose of a PROTAC and its catalytic MoA may result in reduced toxicity.

### iii. Open New Scenarios for Combination Therapies

In 2022, the synergic effect of approved anticancer drugs with **2** and **3** is being tested in clinical trials, laying the foundations for exploiting PROTACs in combination therapies.[36,65] It is known that polypharmacology strategies (i.e., drug combinations or multitarget-directed ligands) for infectious disease are advantageous over single-target treatment.[66] Concurrently, a combination of PROTACs and known inhibitors as well as *multitarget* PROTACs have been recently reported, showing the interest of the medicinal chemistry community in this approach.[67] To note, PROTAC itself, when designed from an existing inhibitor, might act as a multitarget ligand, i.e., inhibitor and/or degrader based on the prevalence of binary


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ACS Bio Med Chem Au 2023, 3, 32−45

interactions that might outcompete the formation of the ternary complex.[68]

Thus, it seems feasible to start thinking about expanding the opportunities found by PROTACs in oncology to other diseases facing similar difficulties.

## TARGETED PROTEIN DEGRADATION IN INFECTIOUS DISEASES

Based on what has been discussed above, the application of TPD technology appears to be an advantageous possibility for the development of anti-infective agents. Indeed, PROTACs, the most studied TPD modality, have begun to be applied in viral diseases in the past few years, leading to selective and effective preclinical prototypes in a short time.[28]

In the following sections, we will first highlight selected PROTAC case studies applied to viral infections. We will then describe the first-in-class antibacterial PROTACs. Finally, we will discuss how the field of small-molecule mediated protein degradation may be exploited in parasitic diseases. Since no antiparasitic PROTAC has been reported yet, we intend to describe the parasite proteasome system in view of future applications.

## PROTACS IN VIRAL DISEASES

As anticipated, the application of TPD technology in infectious diseases mostly encompasses viral infections, with PROTAC modality as the main protagonist. To date, studies have reported the hijacking of the host cell degradation system against different classes of viruses, by exploiting the reported human E3 ligase small-molecule ligands and the concurrent advances in computational techniques for PROTAC design. In 2014, the development of a peptide-based degrader capable of inducing the degradation of hepatitis B virus (HBV) X-protein, an essential target for virus infection, was reported.[69] This first example does not rely on a "conventional" PROTAC structure. Such a peptide-based degrader was constructed by fusing the N-terminal oligomerization and the C-terminal instability domains of the X-protein. Then, the incorporation of a polyarginine cell-penetrating peptide made it cell-permeable. It was predicted that the oligomerization domain would have bound the X-protein, and that the instability domain would have caused the X-protein to be targeted for proteasomal degradation.

In 2019, the first small-molecule-based PROTAC, named DGY-08-097 (**5**, Figure 4) and able to promote the degradation of hepatitis C virus (HCV) NS3/4A protease, was developed.[68] PROTAC **5** was designed starting from the derivatization of the solvent-exposed pyrazine ring of telaprevir, an HCV NS3/4A protease inhibitor withdrawn from the market due to resistance issues. Once identified the suitable attachment point, a PEG linker was used to conjugate the POI ligand to a novel CRBN E3 ligase ligand. This new tricyclic imide moiety was reported to have a greater affinity toward CRBN and to not induce degradation of IMiD neosubstrates. Importantly, **5** retained antiviral activity against telaprevir-resistant viruses (NS3 variants bearing the V55A or A156S mutations), proving the potential of PROTAC modality to overcome drug resistance compared to traditional inhibitors. Furthermore, this study represented the first approach of CRBN-recruiting PROTACs applied to the antiviral field and highly supported the idea of "recycling" withdrawn drugs.

Framed in the COVID-19 pandemic scenario, the application of TPD technology was proposed as a promising strategy to treat and protect the general population.[70] In 2020, a study reported the computer-aided design of peptide PROTACs directed to the RBD-sfGFP complex, a receptor binding domain of the spike protein.[71] Another computational approach based on protein−protein docking and molecular dynamic simulations described the design of telaprevir-based PROTACs, featuring pomalidomide as a CRBN E3 ligase binder and aimed at inducing SARS-Cov-2 Mpro degradation.[72] Nevertheless, it should be noted that those studies were merely based on computational approaches, with no experimental support.

Also driven by the COVID-19 emergency, indomethacin (INM)-based PROTAC **6** (Figure 4) was designed and synthesized.[73] The E3 ligase VHL ligand was linked to the nonsteroidal anti-inflammatory drug INM as a POI ligand. INM inhibited host proteins (i.e., cyclooxygenases-1/2 and human prostaglandin E synthase type 2, possibly implicated in the interaction with the virus), rather than a viral one. **6** demonstrated antiviral activity against a panel of human coronaviruses (HCoV-OC43 and HCoV-229E) and different strains of SARS-CoV-2 (SARS-CoV-2/NL/2020 and SARS-CoV-2/Padova/2021), and its effectiveness was greater than that of INM. However, the intended PROTAC-mediated TPD was not demonstrated.

In 2021, a cyclin-dependent kinase (CDK)-based PROTAC, THAL-SNS032 (**7**, Figure 4), with antihuman cytomegalovirus (HCMV) activity was developed.[74] PROTAC **7** exhibited an experimentally verified degradative mechanism and presented a measurable advantage over the non-PROTAC parent inhibitor. In fact, the anti-HCMV activity of **7** is 4-fold greater than that of the parent compound together with a broader antiviral profile. It also showed a synergistic effect in combination treatment with CDK inhibitors, supporting the use of PROTACs in the drug combination regimen to improve efficacy.[74]

In 2022, the metabolite APL-16-5 (**8**, Figure 4) was discovered to exert antiviral activity against influenza A virus (IAV) by binding the E3 ligase TRIM25 and thus promoting the degradation of the IAV polymerase through ubiquitination.[75] Although this is a noncanonical rationally designed PROTAC, it supports the use of the TPD modality against influenza viruses.

In the same year, another study described the development of oseltamivir-based PROTACs with anti-H1N1 influenza activity, considering both VHL and CRBN E3 ligase ligands.[76] The best PROTAC **9** (Figure 4), featuring the VHL binder, degraded the neuraminidase (NA) protein through UPS and exhibited potent antiviral activity toward both the wild-type H1N1 virus and an oseltamivir-resistant strain.

In 2022, a new class of pentacyclic triterpenoid-based PROTACs has been discovered as hemagglutinin (HA) degraders.[77] As for the POI ligand, an oleanolic acid derivative, an active plant metabolite with anti-influenza A/WSN/3 virus activity, was chosen. Both VHL and CRBN E3 ligase binders were considered. PROTACs were optimized based on different linker lengths to enhance the formation of a productive ternary complex by means of computational tools. The most promising PROTAC **10** (Figure 4) was also able to promote HA degradation in cellular models.[77]

Collectively, the applicability of the PROTAC modality to different classes of viruses seems extensively demonstrated.


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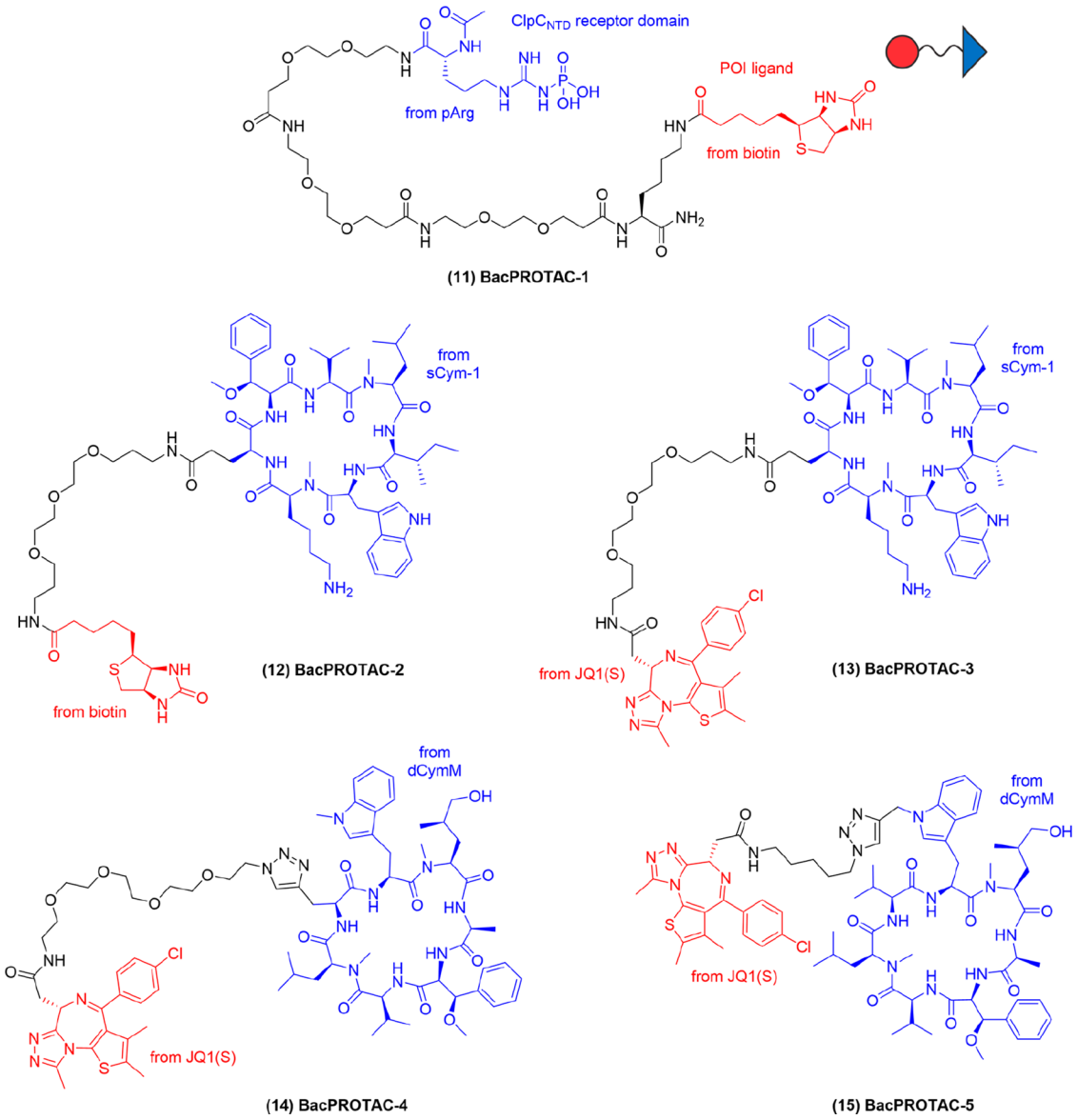


**Figure 5.** BacPROTACs1−5 (**11**−**15**).

This is mainly because viruses hijack the human UPS, which has been characterized and for which small-molecule E3 binders are already available. Potential advantages with respect to conventional drugs regard improving the antiviral activity, overcoming drug resistance, and "recycling" withdrawn compounds. In addition, PROTACs have been demonstrated to be promising in combination treatments.

## PROTACS IN BACTERIAL DISEASES

PROTAC-mediated TPD applications in bacterial diseases are not far behind. Even though the concept of using the cellular protein degradation machinery is restricted to eukaryotes, in prokaryotes there are possibilities that could help to translate the TPD technology against these infective agents in an innovative fashion. A 2022 landmark publication described the development of what has been dubbed "BacPROTACs".[29] Such small-molecule degraders are based on an innovative approach inspired by PROTAC modality that consists of binding to the substrate receptor of the bacterial ClpC:ClpP (ClpCP) protease, priming neo-substrates for degradation. In this way, BacPROTACs harness the bacterial degradation machinery to promote a selective degradation of pathogen-specific proteins, like a "Trojan horse". Phosphorylated arginine residues (pArg) serve as a degradation signal that is recognized by ClpCP. As a first proof of concept, BacPROTAC-1 (**11**, Figure 5), featuring a pArg moiety, was able to promote the selective degradation of a model protein. The monomeric streptavidin (mSA) neo-substrate was used, and it turned out to be recruited by the biotin moiety (high affinity mSA ligand). To better understand the influence of substrate-specific properties on ClpCP activity, various mSA fusion proteins (*B. subtilis* targets: NrdI, TagD, NusA, and Kre) were cloned, and **11**-mediated degradation activity was assessed. Subsequently, considering that the ClpC1P1P2 protease (present in Mycobacteria) has a functional pArg receptor site fully conserved in its ClpC1$_{NTD}$ domain, the authors extended this approach to the *Mycobacterium smegmatis* ClpC1 system. In that analysis, **11** promoted the proximity and the formation of a ternary complex with mSA and ClpC1$_{NTD}$. To overcome the PK limitations of pArg-based


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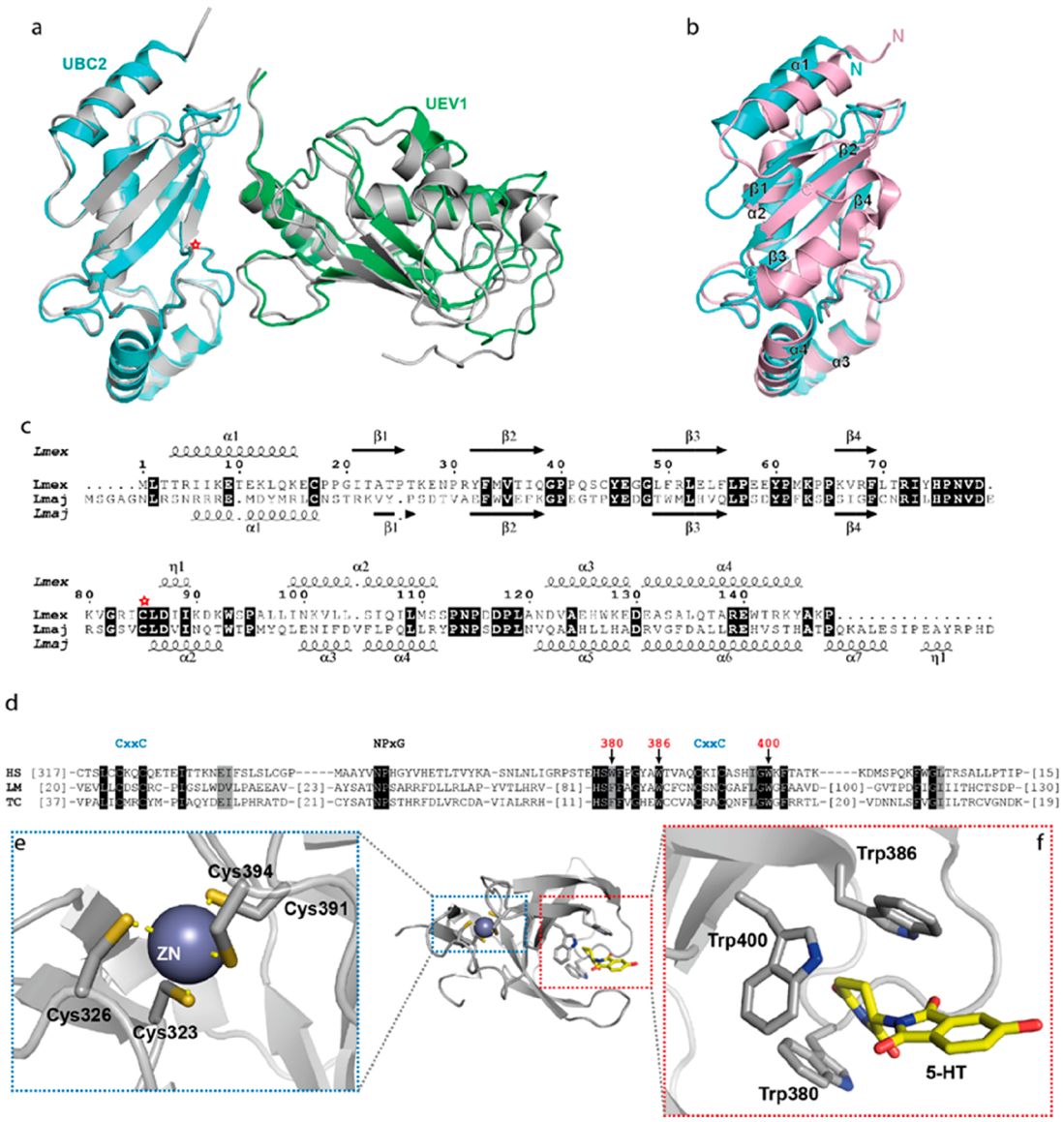


**Figure 6.** Structural characterization of *Leshmania* E2 enzyme and conservation of the thalidomide binding domain in Kinetoplastida. (a) Superimposition of UBC2-UEV1 heterodimer (cyan/green) with the human homologue complex UBE2N−UBE2 V2 (gray, PDB id: 1J7D). (b) Structural alignment of *L. mexicana* E2 UBC2 (cyan, PDB id: 6ZM3) and *L. major* uncharacterized E2 (pink, PDB id: 1YF9). RMSD of two aligned structures is 1.26 Å. The N and C termini of each protein are reported. The secondary structure of *L. mexicana* UBC2 is indicated as in Burge et al.[80] (c) Sequence alignment of *L. mexicana* E2 UBC2 (Lmex) and *L. major* uncharacterized E2 (Lmaj). The secondary structure of both sequences is indicated. Red star evidences the catalytic Cys residue (Cys85). (d) Sequence alignment of human CULT domain (HS, NP_057386.2) to *L. major* and *T. cruzi* homologues (LM XP_001681231.1 and TC EKG02463.1 respectively). The Zn binding motif and tri-Trp cage are reported on the top of the alignment. (e and f) 3D representation of Zn and thalidomide binding sites. The structure of human CRBN protein (gray) bound to 5-hydroxy-thalidomide (yellow) was used (PDB id: 7bqv).[96] All images were prepared with PyMOL, and sequence alignments were represented with ESPript 3.0.

PROTACs, cyclomarin A (CymA)-like cyclic peptides (sCym-1 and dCymM), well-characterized $ClpC1_{NTD}$ antibiotics, were used. CymA were conjugated to JQ1 as a POI ligand, to provide BacPROTAC-2−5 (**12**−**15**, Figure 5), able to degrade in *in vitro* assay bromodomain-1 (BD1) of BRDT as an attractive model substrate. Nevertheless, the reduced *in vivo* degradation efficiency of BacPROTACs compared to their *in vitro* data is more likely depending on poor cellular permeability. Another drawback is related to the use of different fusion proteins which may affect degradation efficiency, but it was instrumental for preliminary proof-of-concept studies. Definitely, this article laid the foundation for an innovative PROTAC application that could lead to the development of a new class of antibacterial degraders. Besides that, the collected findings related to the structural biology of activated ClpCP support the idea that PROTACs can be used as chemical probes for basic biology or target validation endeavors.[78]

## PROTACS IN PARASITIC DISEASES: DREAM OR REALITY?

Protozoan parasitic diseases, such as malaria caused by *Plasmodium falciparum*, leishmaniasis, and trypanosomiases caused by Trypanosomatids, have limited treatment options, despite the comprehensive effort of public−private scientific


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ACS Bio Med Chem Au 2023, 3, 32−45

partnerships over the past few years.[79] This scenario encourages the search for innovative therapeutic alternatives, such as those embraced by the TPD. PROTACs, as previously discussed, could provide promising opportunities to overcome the current issues of antiparasitic drug discovery, e.g., drug-resistant parasite strains and modulation of "undruggable" targets. However, as far as we know, no PROTAC has been developed for this therapeutic indication. This may be due to the fact that still little is known about the UPS in protozoan parasites. Thus, in the following paragraph, we will provide an overview of the parasitic degradation system.

## UPS IN PROTOZOAN PARASITES

Most of the UPS components in protozoan parasitic species have been extensively characterized through comparative genomic analysis.[80−82] In silico analyzed data suggest an overall conservative tendency among the E1 and E2 enzyme pools, while the E3 ligase arsenal is the most abundant and widely differentiated. Kinetoplastid *Trypanosoma* and *Leishmania* species have two E1 UBAs, about 15 E2 ubiquitin-conjugating (UBC) enzymes, and a variable number of E3 ubiquitin ligases ranging from 50 to 80.[80] These numbers are confirmed for the apicomplexan parasites, except for the E1 UBA1 enzyme, for which only one type has been identified in *Plasmodium falciparum*.[82] For reference, in humans, two E1s (UBA1 and UBA6), 40 E2s, and over 600 E3s exist, whereas *S. cerevisiae* has one E1, 11 E2s, and 60−100 E3s.[83] Moreover, 20 DUBs have been bioinformatically identified in *L. mexicana*,[84] a number compatible with that of *S. cerevisiae*. In humans, around 100 DUBs were found.[85] Two variants of the UBA1 enzyme, namely, UBA1a and UBA1b, have been characterized as functional ubiquitin-activating enzymes in *T. brucei* and *L. mexicana*.[80,86] Both are more closely related to the human UBA1 than UBA6. However, a species specificity at the adenylation site exists since *T. brucei* UBA1 and *L. major* UBA1 were demonstrated to be resistant to the potent human UBA1 inhibitor TAK-243.[86] Therefore, the trypanosomatid UBA1 enzyme has been discussed as a potentially druggable target.[87] This is not true for the malaria parasite, whose UBA1 enzyme was efficiently inhibited by the TAK-243 molecule.[88] In *P. falciparum*, the components of the degradation pathway associated with the endoplasmic reticulum (ER) were characterized as essential for the parasite's survival.[89] The promiscuous cytosolic PfUBA1 and PfUBC7 proteins were demonstrated to interact with ER-associated E3 ligase HRD1, recruiting the proteins in ER addressed to the degradation pathway.[89] Recently, a study about an extensive mutational analysis of E1, E2, and E3 genes in *L. mexicana* allowed the discovery that several UPS components are important for the organism's survival and differentiation. Indeed, null mutants were successfully generated for almost all identified genes, except for the E1 UBA1 and three E2s, suggesting them to be essential in promastigotes. The two E1s, UBA1a and UBA1b, were previously demonstrated to be essential in *T. brucei*.[90] In more detail, inactivation of E2 enzymes UBC2 and UEV1 caused the most severe phenotypes in all of the differentiative stages. The structure of the UBC2−UEV1 heterodimer has been solved at 1.7 Å (PDB ID: 6ZM3;[80] Figure 6a). UBC2 was confirmed to be an active E2 enzyme, able to covalently bind a Ub unit, whereas UEV1 is an inactive as E2, but it is supposed to regulate UBC2 activity.[80] The protein−protein interaction and heterodimeric structure were previously described for the human homologue couple UBE2N−UBE2 V2 (PDB ID: 1J7D;[91] Figure 6a) and yeast Ubc13−Mms2 (PDB ID: 1JAT).[92] The structure of *L. mexicana* UBC2 is similar to that of *L. major* E2 (PDB ID: 1YF9 [unpublished]) with which shares 36% of the sequence (Figure 6b,c). The viability of *T. brucei* E2 mutants has been also assessed.[90] Alsford and co-workers have shown that the knockdown of the parasite ortholog of the human UBE2D (UbcH5) and UBE2N (Ubc13), sharing the highest % identity among the E2s, caused respectively a 77% and 87% reduction in viability, indicating their central role in the ubiquitylation in *T. brucei*.[90] The functional characterization of the *T. brucei* CDC34 homologue sharing only 22% identity with the human E2 enzyme demonstrated its in vitro ubiquitin-conjugating activity;[93] moreover, its knockdown led to a slower population growth rate. The role of some E3 ligases and E3 ligase complexes in cell cycle progression and organism differentiation has been explored in *T. brucei*. The down-regulation of the trypanosomal homologue components of the well-studied SCF complex generated different phenotypes, which could indicate that they might not be functioning as a stable complex as it was demonstrated for *S. cerevisiae*.[93] Down-regulation of the SKP1 component revealed its conserved role in the G1/S transition, while the lack of an RBX1 component (i.e., the RING E3 ligase interacting with CDC34 E2) interferes with the kinetoplast DNA replication. The depletion of the CULLIN1 unit, on the other hand, did not determine any phenotype, indicating its redundancy with other cullins.[93] The components of the anaphase promoting complex/cyclosome (APC/C) have been experimentally identified in *T. brucei* cellular extract through LC-MS/MS. The RNAi knockdown of one of the components, AP2, caused the mitotic arrest and the polyubiquitylated cyclin B accumulation.[94] This demonstrated a certain degree of conservation of APC/C function, despite the high divergence observed between *T. brucei* and *S. cerevisiae* complexes.

Despite playing a crucial role in the development of PROTAC, the functional studies on parasite E3 ligase complexes are poorly supported by structural studies. The opportunity to harness the human E3 ligase targets structurally defined and used for the most advanced PROTACs, such as CRBN or VHL, appears to be tempting for the development of small-molecule TPD-based therapeutics for parasitic diseases.

In Kinetoplastida, a protein containing the CRBN thalidomide binding domain, annotated as the CULT domain, has been bioinformatically identified.[95] The domain is conserved among several *Leishmania* and *Trypanosoma* species (Figure 6d). The overall fold and the zinc coordination site are conserved (Figure 6d,e), while the thalidomide binding site has some distinctions when compared to the human CULT domain (Figure 6e,f). The most striking one is the substitution of one Trp residue with a Phe in the tri-Trp cage binding the glutarimide ring of thalidomide (Figure 6e,f). The experimental characterization of the kinetoplastid CULT domain containing protein as part of the ubiquitylation machinery would open the possibility for the readaptation of the thalidomide scaffold for the PROTAC development for leishmaniasis or trypanosomatid diseases.

## TPD TECHNOLOGIES IN PARASITIC DISEASES

Related to the application of TPD technologies in parasitic diseases, an interesting attempt in a yet-to-be-published study has been reported this year.[97] The adaptation of the degraded green fluorescent protein (deGradFP) system has been used to


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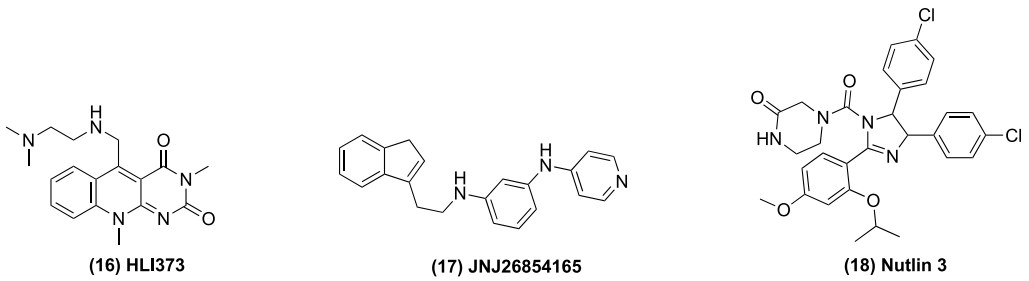


**Figure 7.** *P. falciparum* E3 ligase inhibitors **16**−**18**.

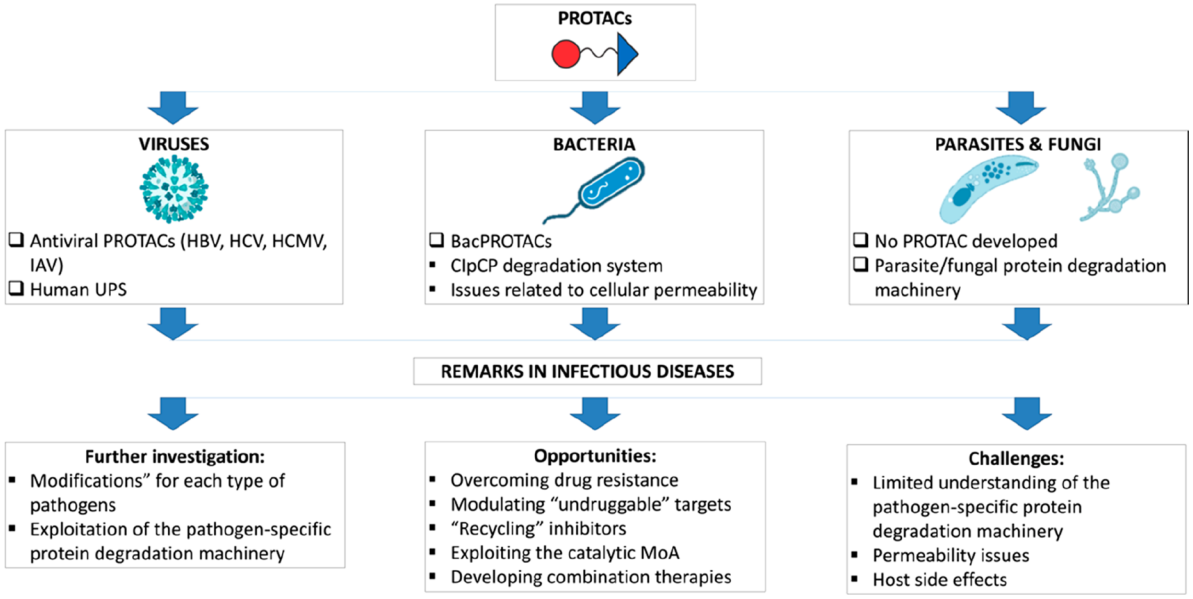


**Figure 8.** Outlook of TPD applications to infectious diseases.

induce the depletion of green fluorescent protein (GFP)-fusion proteins in *T. brucei*. The degrader deGradFP features Vhh-GFP4 (an anti-GFP nanobody) fused to the F-box protein, which is a substrate recognition for the SCF complex. Subsequently, GFP-fusion proteins are ubiquitinated by the SCF E3 ubiquitin ligase complex. Given the ease in making GFP fusion cell lines in *T. brucei*, deGradFP can serve as a powerful tool to rapidly deplete POIs and a way to validate parasite-specific E3 ligase structure and functionality. Interestingly, FBP75 was identified as a domain of the trypanosomatid SCF complex that could serve as an anchor point to promote protein degradation. However, the authors have still to evaluate (i) the proteasome-mediated degradation, (ii) "off-target" effects, and (iii) the interaction sites between the FBP75 with Skp1 or cullin proteins, which could lead to the development of functional degron systems applied to *T. brucei*.

Along the same line, other targeted degradation tools have been previously applied to *Leishmania* involving degrons and fusion proteins.[98,99] These studies were based on genetic manipulation of target genes and will not be discussed in this Perspective.

Studies devoted to discovering new E3 ligase candidates both in the host and in the pathogen will advance PROTAC modality also in antiparasitic drug discovery. For instance, in 2022, the role of the host E3 ligase RNF123 in a rodent malaria infection has been disclosed.[100] Another study found the host E3 ligase MARCH1 involved in the regulation of antimalarial immunity.[101] Additionally, in 2020, 28 enzymes belonging to the ubiquitin system of *Leishmania* proteasome have been described, and the UBC2-UEV1 E2 complex appeared to be implicated in differentiation processes.[80]

To date, some studies highlighted the UPS and the proteasome as a potential drug target in malaria and kinetoplastid diseases.[102] In 2017, known E3 ligase inhibitors (HLI373, JNJ26854165, and NSC6811, **16**−**18**, Figure 7) were phenotypically screened on *P. falciparum*. Although no proof for binding and/or structural information was provided, this work raises interesting clues for recruiting parasite-specific E3 ligase.[103] In 2020, COP9 signalosome was found to be an essential and druggable parasite target that regulates protein degradation.[104] Remarkably, the study on the parasite-specific UPS proteins could contribute to novel TPD application and also to the discovery of novel therapeutic targets.[81]

Collectively, the application of PROTACs' modality for the development of antiparasitic agents could provide different advantages. However, the applicability of this modality needs to be proved, since, to the best of our knowledge, there are no reported antiparasitic PROTACs. The exploitation of the parasites' own protein degradation system by PROTACs needs to face some issues beforehand: (i) suitable E3 ligases in parasite degradation systems, taking into account similar host E3 ligases to avoid loss of specificity, (ii) POIs in different stages of the parasite life cycle, and (iii) different localizations across life stages. In fact, parasite persistence in different cells, parasite reservoirs in inaccessible tissues, complex life cycles,


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ACS Bio Med Chem Au 2023, 3, 32−45

and different parasite stages could be potentially addressed by PROTAC modality.

Furthermore, potential disadvantages cannot be ruled out. As described above, the parasitic degradation pathway is similar to the human pathway. Thus, in the development of a new antiparasitic PROTAC, side effects should be carefully considered and early evaluated. Drug developers should be aware that, although potentially promising, it will be challenging to develop an antiparasitic degrader not causing issues for the human host.

## OUTLOOK

The beginning of the "modern antibiotic era" is linked with the name of Paul Ehrlich. Ehrlich's idea of a "magic bullet" that selectively modulates only microbe-specific targets and not host-related ones was based on an observation that synthetic dyes could stain specific microbes but not others. Ehrlich envisioned the possibility of identifying compounds "able to exert their full action exclusively on the parasite harbored within the organism".[1]

Could PROTACs be a "new magic bullet" to fight infectious diseases? The idea of selectively modulating protein levels could enlarge the anti-infective drug discovery landscape, which classically encompasses compounds designed to inhibit pathogen specific protein functions. Antiviral and antibacterial applications seem promising and could be extended to other infectious diseases responsible for millions of deaths globally each year, such as parasitic and fungal infections.

Because of their peculiar and catalytic MoA, anti-infective PROTACs might be advantageous in terms of efficacy, toxicity, and selectivity. In fact, a PROTAC may in principle harness both the host and the pathogen protein degradation machinery, resulting in a more effective, selective, and safe treatment. Importantly, PROTACs may also overcome the emergence of AMR, as already demonstrated in antiviral PROTACs. Furthermore, PROTACs might advance anti-infective drug discovery by (i) covering "undruggable" targets, (ii) "recycling" inhibitors from classical drug discovery approaches, and (iii) opening new scenarios for combination therapies (Figure 8). Still, the medicinal chemistry community has to face many challenges: (i) limited understanding of the pathogen protein degradation machinery, (ii) PROTAC penetration through pathogen-specific membranes (e.g., Gram-negative bacteria cell wall or glycosomal compartmentalization in Trypanosomatidae), (iii) different pathogen localizations across life stages (e.g., *Leishmania* amastigote in host macrophages or malaria merozoites in erythrocytes), and (iv) host side effects.

One can be skeptical about the effectiveness of TPD in infectious diseases, but some cards are on the table, and we must play the game.

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## ABBREVIATIONS

APC/C, anaphase promoting complex/cyclosome; BET, bromodomain and extraterminal protein; CDK, cyclin-dependent kinase; CRBN, cereblon; DUBs, deubiquitylating enzymes; FSE, frameshifting element; HA, hemagglutinin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCMV, human cytomegalovirus; HCV, hepatitis C virus; IAP, inhibitor of apoptosis protein; IAV, influenza A virus; IMiDs, immunomodulatory drugs; INM, indomethacin; MDM2, mouse double minute 2; MoA, mechanism of action; NB, neuroblastoma; mSA, monomeric streptavidin; NCE, new chemical entities; PD, pharmacodynamics; PK, pharmacokinetics; POI, protein of interest; PROTACs, proteolysis-targeting chimeras; PTD, proteasome-targeting domain; RNase, ribonuclease; SCF, Skp1-Cullin-F box; TPD, targeted protein degradation; UBA, ubiquitin-activating enzyme; UBA1, ubiquitin-activating enzyme E1; UBC, ubiquitin-conjugating



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ACS Bio Med Chem Au 2023, 3, 32−45

enzyme; UPS, ubiquitin proteasome system; VHL, Von Hippel−Lindau; WHO, World Health Organization.

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