PROTAC: targeted drug strategy. Principles and limitations*

O. A. Koroleva,[a] Yu. V. Dutikova,[b] A. V. Trubnikov,[a] F. A. Zenov,[a] E. V. Manasova,[a]
A. A. Shtil,[a,c] and A. V. Kurkin[a]★

[a]*Department of Chemistry, Lomonosov Moscow State University,*
Build. 3, 1 Leninskie Gory, 119991 Moscow, Russian Federation.
Fax: +7 (495) 939 2288. E-mail: indolester@gmail.com
[b]*Patent & Law Firm "A. Zalesov and Partners",*
Build. 9, 2 ul. Marshala Rybalko, 123060 Moscow, Russian Federation.
E-mail: Dutikova@azalesov.com
[c]*N. N. Blokhin National Medical Research Center of Oncology,*
Ministry of Health of the Russian Federation,
Build. 15, 24 Kashirskoe shosse, 115478 Moscow, Russian Federation.
E-mail: shtilaa@yahoo.com

The PROTAC (*PROteolysis TArgeting Chimera*) technology is a method of targeting intracellular proteins previously considered undruggable. This technology utilizes the ubiquitin—proteasome system in cells to specifically degrade target proteins, thereby offering significant advantages over conventional small-molecule inhibitors of the enzymatic function. Preclinical and preliminary clinical trials of PROTAC-based compounds (degraders) are presented. The review considers the general principles of the design of degraders. Advances and challenges of the PROTAC technology are discussed.

Key words: PROTAC technology, proteolysis, cancer therapy.

The concept of targeted therapy has gained popularity since the introduction of gleevec (imatinib) into the clinic for the treatment of cancer (in the 1990s). This approach is based on the fundamental knowledge of the biological mechanism underlying particular disease pathogenesis, as well as the possibility of targeted inactivation of this mechanism for therapy. The principal idea is that this targeting eliminates the pathogenic factor with minimum damage of intact cells. Due to the progress in medicinal chemistry and related disciplines, clinicians are armed with dozens of targeted drugs, and hundreds of compounds are currently under clinical trials. The majority of these agents target proteins with enzymatic properties, such as protein kinases, epigenetic markers, *etc*. The structures of these targets were studied in detail, making it possible to synthesize large target-focused compound libraries and identify lead compounds with high inhibitory activity and selectivity. The inactivation of nonenzymatic proteins is more challenging. Among these are important elements of signaling cascades, numerous structural proteins,

transcription factors, *etc*. The PROTAC (*PROteolysis TArgeting Chimera*) technology was invented to address this problem. This methodology is based on the design of small-molecule bifunctional structures, which have a binding affinity for the target and bind to an ubiquitin ligase. Such conjugates mediate the transport of a supramolecular complex to the proteasome and protein hydrolysis. The review considers the general principles of the design of chemical tools for PROTAC, presents protein candidates for targeted proteolysis, and discusses advances and methodological challenges for the development of this technology.

1. Development of the problem

The ubiquitin—proteasome system (UPS) is the major mechanism utilized by eukaryotic cells to degrade intracellular proteins.[1,2] The attachment of several ubiquitin molecules to the target protein is required for the activation of the UPS (Fig. 1). The protein ubiquitination involves the E1—E2—E3 enzyme cascade. The ubiquitin-activating enzyme E1 uses adenosine triphosphate (ATP) to activate ubiquitin *via* adenylation. Then adenylated ubiquitin is conjugated to the ubiquitin-

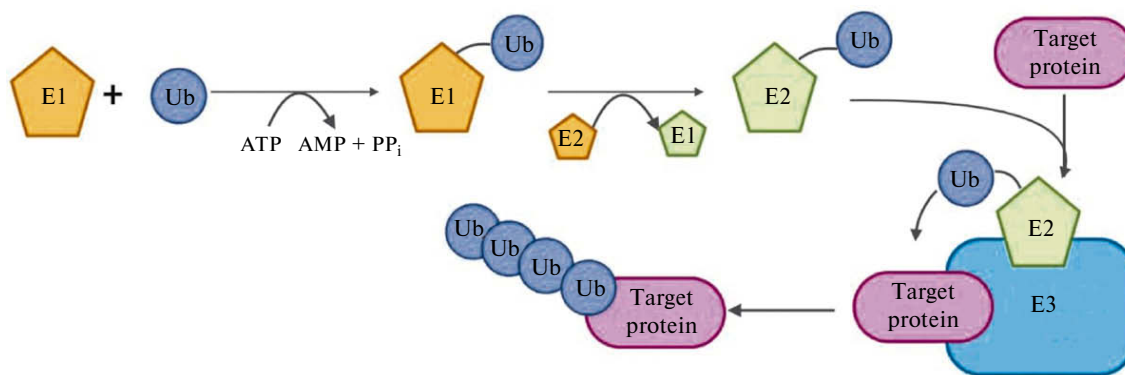* Dedicated to the blessed memory of Academician of the Russian Academy of Sciences N. S. Zefirov.

Fig. 1. Mechanism of ubiquitination of the target protein. Ub is ubiquitin, ATP and AMP are adenosine triphosphate and adenosine diphosphate, respectively, PP_i is inorganic pyrophosphate.

conjugating enzyme E2. The activated ubiquitin is then transferred by the E3 ubiquitin ligase from E2 to a lysine residue on the surface of the target protein. Ubiquitin contains seven lysine residues, all of which can be ubiquitinated by E3 ligase to form polyubiquitin chains. Polyubiquitinated proteins are recognized and degraded by the 26S proteasome, which is a large complex containing six proteolytic sites. The selection of proteins for degradation by ubiquitin is dictated by the *N*-terminal amino acid residue because the carboxyl group of the *C*-terminal amino acid residue of ubiquitin is bound either to the *N*-terminal amino group (peptide bond) or to the side-chain ε-amino group of a lysine residue of the target protein (isopeptide bond).

The PROTAC technology is based on the ubiquitin-mediated proteolysis of proteins. The PROTAC molecules are small-molecule chimeras that bind the target protein to label it with ubiquitin followed by the proteasomal degradation.[3–28] These heterobifunctional molecules consist of a ligand for the target protein (*protein of interest* (POI)), a ligand for E3 ligase (E3 ligase binder), and a linker (generally, a short structurally simple alkyl or polyethylene glycol (PEG) chain)[29] (Fig. 2).

The PROTAC molecules induce the target protein—PROTAC—E3 ligase ternary complex formation, recruiting the E3 ubiquitin ligase, a component of the UPS, into proximity with the target. This promotes the
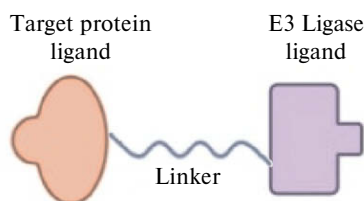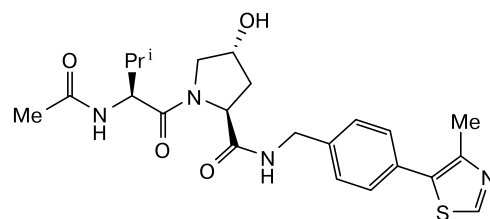
ubiquitination of the target and, finally, its proteasome-mediated hydrolysis (Fig. 3).

To date, four small molecule-based E3 ligase-binding motifs are commonly used for the development of PROTACs.[4] The anticancer drug thalidomide and its derivatives, which serve as ligands for the protein cereblon (CRBN), are most frequently used for this purpose.[30] It is the binding to E3 ubiquitin ligase that is responsible for the effect of thalidomide and its close analogs. This discovery and the determination of the crystal structure of the thalidomide—CRBN complex in 2014[31] allowed the design and investigation of PROTACs targeting CRBN-mediated ubiquitination, thereby triggering the degradation of oncoproteins.[30,32,33]
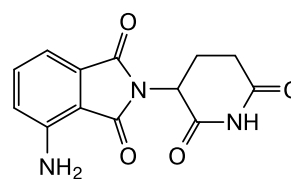
Thalidomide

Ligands for the von Hippel—Lindau (VHL) E3 ubiquitin ligase[34–37] are the second most used group. Due to the efficient and potent degradation of a broad range of POIs and the ability to bypass target-independent effects of CRBN, the VHL ligand is commonly used for the E3 ligase binding, along with CRBN ligands.[3,5,38] As an example, we refer to PROTAC VHO32.

Target protein ligand   E3 Ligase ligand

Linker

Fig. 2. Schematic representation of the structure of a PROTAC molecule.
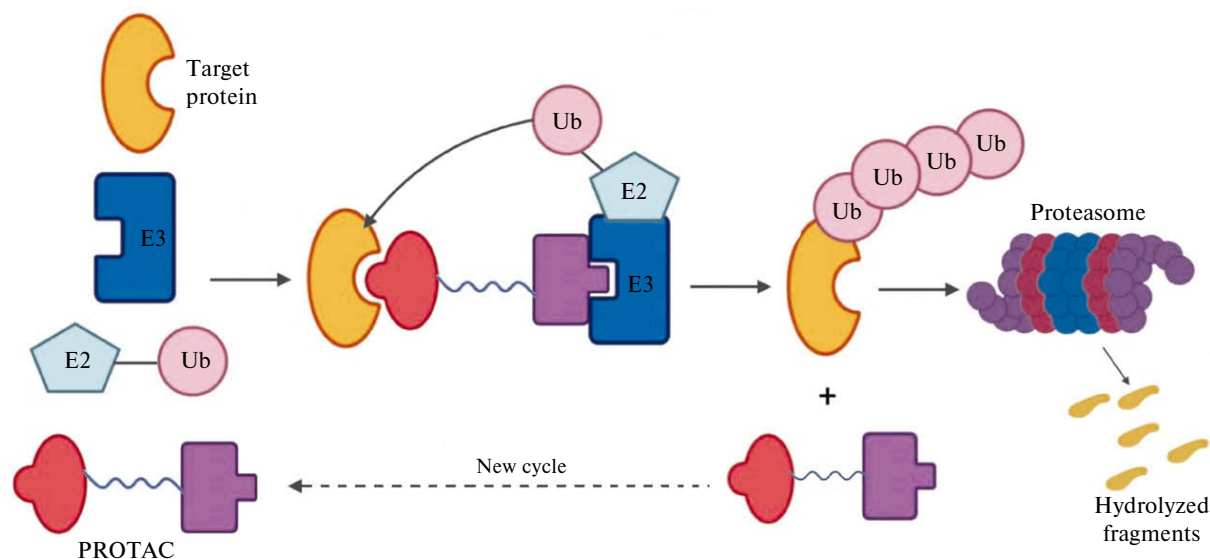

VH032

Fig. 3. Mechanism of PROTAC technology (the figure is adapted from the publication[3]). The PROTAC molecules (degraders) consist of two functional parts: a target protein ligand (marked in pink color) that binds the target protein and an E3 ubiquitin ligase ligand (violet). The target protein—PROTAC—E3 ligase ternary complex formation induces ubiquitination of the target protein, resulting in its proteasome-mediated hydrolysis. The PROTAC molecules can dissociate from the ternary complex and trigger several cycles of degradation.

Ligands for the inhibitor of apoptosis proteins (IAP), *e.g.*, bestatin and its more complex derivatives, such as LCL-161, are also applied in PROTAC technology.[39–41] Degraders based on ligands for IAP (*specific non-genetic IAP-based protein erasers*, SNIPERs) often induce the less pronounced degradation compared to PROTACs based on ligands for other E3 ligases;[38] the autoubiquitination can occur after the interaction with IAP.[41]


Bestatin

LCL-161
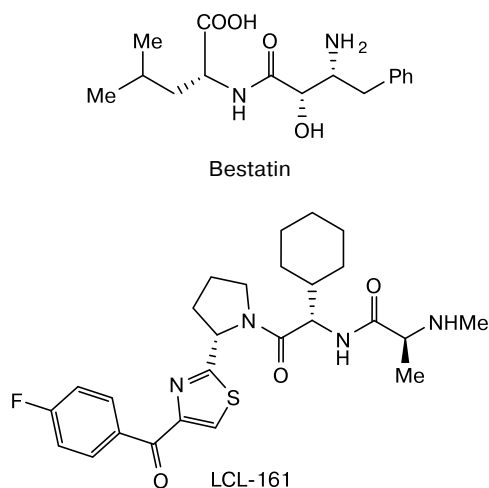
Small molecules that bind to the protein MDM2, which serves as an E3 ubiquitin ligase that regulates the p53 tumor suppressor by promoting its proteasome-mediated degradation, are used less often. The first

MDM2-recruiting PROTAC was synthesized based on a nutlin-3 derivative.[42] After the several-year assessment of the binding of PROTAC to the CRBN and VHL ligands, a potent degrader based on the modified MDM2 ligand was synthesized in 2019.[43] It is worth noting that, despite efforts to design MDM2-recruiting PROTACs, these compounds are less effective than hybrid molecules based on VHL or CRBN ligands.[38,43]


Nutlin-3
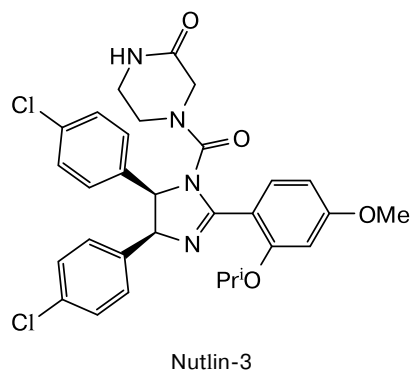
New noncovalently-binding ligands for such E3 ligases as DCAF15 and AhR were identified and used to synthesize PROTACs.[44–50] The indisulam derivative E7820 was utilized as the DCAF15 ligand.[46] It was demonstrated[47] that PROTACs incorporating AhR E3 ligase ligands (*e.g.*, β-naphthoflavone derivatives) can induce the degradation of POI.

The covalent fragment library screening[46] uncovered the CCW-16 fragment as a suitable ligand for the E3
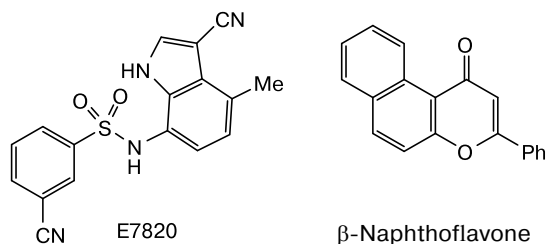
ubiquitin ligase RNF4. A compound based of the triterpenoind bardoxolone was found to be a reversible covalently-binding E3 ligase ligand, which was used to synthesize PROTAC.[48] The natural product nimbolide was utilized as a structural component of PROTAC targeting the E3 ligase RNF114.[49] It was demonstrated that the ligand EN-106 is a covalent recruiter of the E3 ligase FEM1B.[50]

Nimbolide
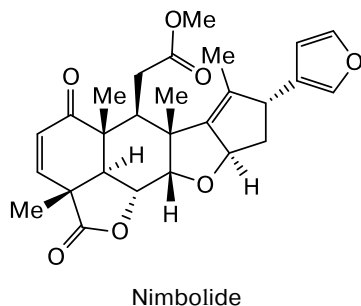
The compound Protac-1 was synthesized and was shown to induce the degradation of methionine aminopeptidase-2 (MetAP-2).[51] In this study, it was demon-

strated for the first time that the chimeric concept as small-molecule chemical tools can be used for the hydrolysis of cellular proteins. Subsequently, peptide-based PROTACs were developed for the proteolysis of androgen receptors (AR), estrogen receptors (ER), aryl hydrocarbon receptors (AHR), and the FK506-binding protein FKBP12.[51-55]
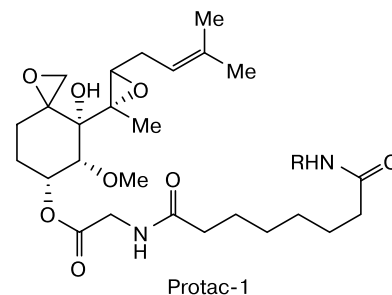
Protac-1

R is GGGGGGGRAEDS*GNES*EGE-COOH or
GGGGGGGDEHDS*GLDS*M-COOH

* Phosphorylated serine.

Since peptides have low lipophilicity (which may hinder the plasma membrane penetration) and are easily hydrolyzed by digestive enzymes, peptide-based PROTACs (bioPROTACs), for example AP21998/HIF1,[52] were poorly accumulated in the cells and proved to be unstable. Therefore, efforts were focused on the development of non-peptidic E3 ligase ligands. The first synthetic cell-permeable small-molecule PROTAC was developed in 2008.[56] This PROTAC

AP21998/HIF1

SARM-nutlin PROTAC

ARD-69

MD-224

ARV-110

ARV-471

DT2216

Navitoclax
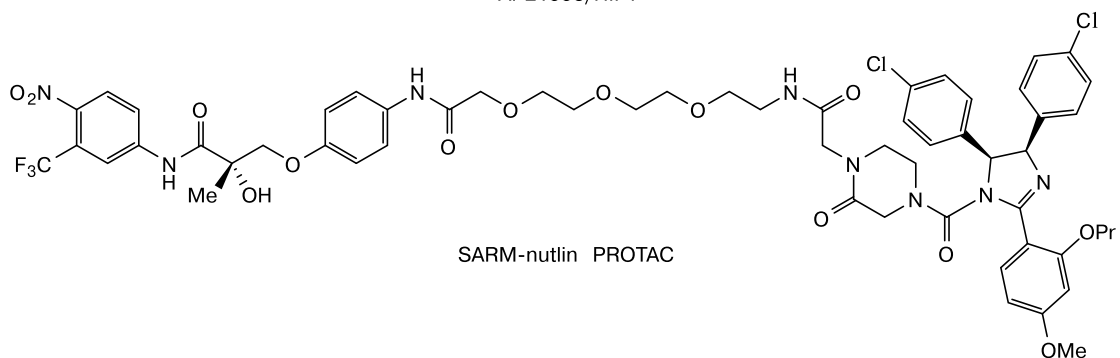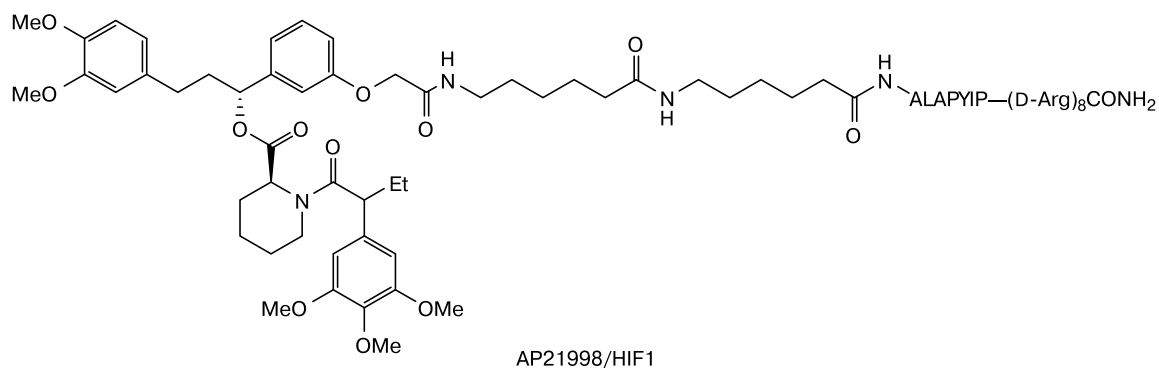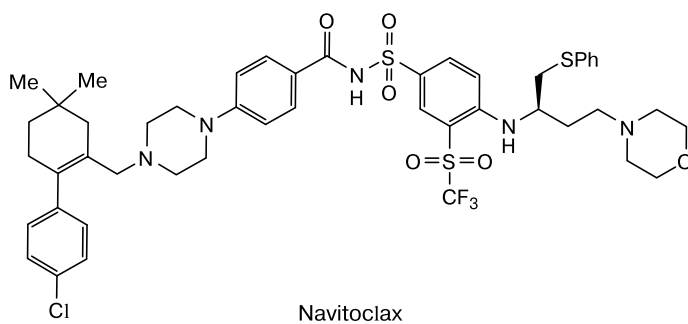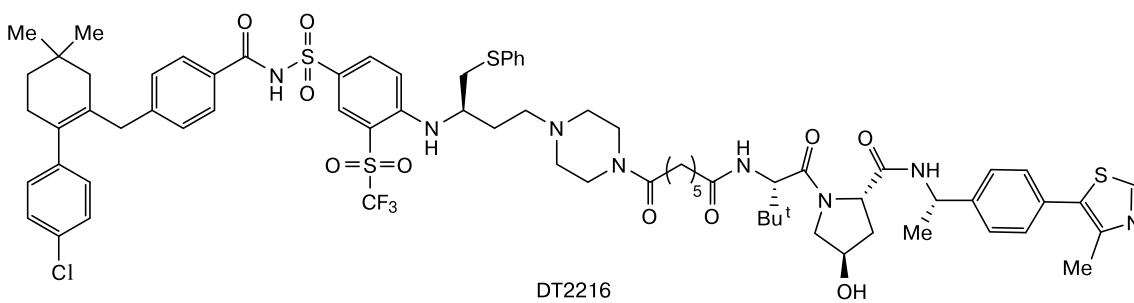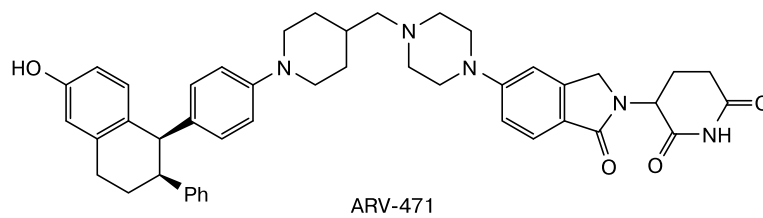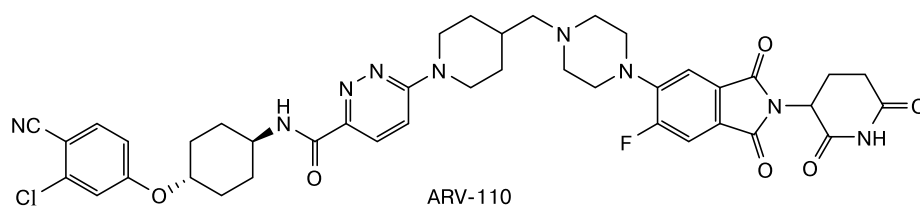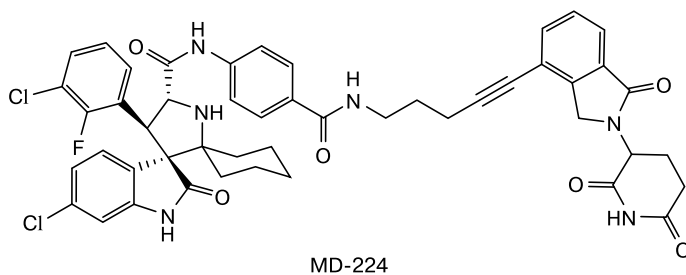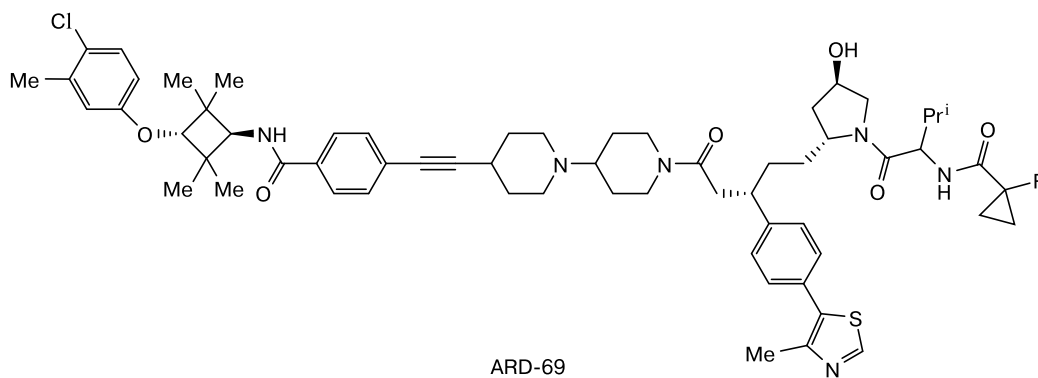
consists of a non-steroidal selective androgen receptor modulator (SARM) ligand and a nutlin fragment (MDM2 ligand) connected by a polyethylene glycol (PEG)-based linker (SARM-nutlin PROTAC). It induced the ubiquitination of the androgen receptor (AR) and its proteasomal degradation.

The PROTAC technology has attracted great interest of both academia and biopharmaceutical industry, resulting in its rapid development. A series of active and highly selective PROTACs were designed.[32,57−59] For example, the PROTACs ARD-69 and MD-224 were synthesized.[57,59]

An important step was the accomplishment of phase I clinical trials of the PROTACs ARV-110 and ARV-471 targeting the proteins AR and ER, respectively (NCT04072952 and NCT03888612), which were developed by Arvinas.[60] This step marked the transfer of the technology from the laboratory to the clinical environment. The compounds ARV-110 and ARV-47 were shown to have a favorable safety profile and they entered phase II clinical trials. Besides, the selective degraders DT2216 and navitoclax, targeting BCR-X_L (NCT04886622), and FHD-609, targeting BRD9 (NCT04965753), *etc.*, entered phase I clinical trials.[61]

The PROTAC technology provided the basis for targeted protein degradation (TPD) strategies. Besides, classes of heterobifunctional molecules, which induce the target protein degradation *via* mechanisms other than the ubiquitin—proteasome system, are emerging. Examples are AUTAC (*autophagy-targeting chimeras*), ATTEC (*autophagosome-tethering compounds*), LYTAC (*lysosome-targeting chimeras*), and AbTAC (*antibody-based PROTACs*). Both AUTAC and ATTEC are MADTACs (*macroautophagy degradation-targeting chimeras*).[62] Like PROTACs, AUTECs and ATTECs trigger TPD in cells, while LYTAC and AbTAC trigger intercellular TPD *via* an extracellular process.[63]

Among processes that recruit a target protein in proximity to a factor inducing its degradation, mo-

lecular glues are worth mentioning. Generally, molecular glues are monomeric small molecules (without a linker), which can simultaneously bind a target protein and an E3 ligase, enabling the ubiquitination and proteasome-mediated degradation of the target protein. Synthetic molecular glues include lenalidomide, thalidomide, and pomalidomide.[64,65] These compounds promote the formation of a complex of CRBN with the transcription factors IKZF1 and IKZF3, inducing the ubiquitination of the latter and their proteasomal hydrolysis. Since synthetic molecular glues are CRBN E3 ligase ligands, they are used for the construction of PROTACs.[64,65]
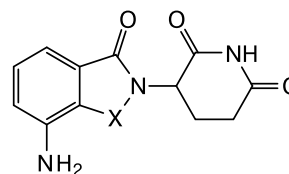
Pomalidomide (X = C=O)
Lenalidomide (X = CH₂)

The PROTAC technology is also of interest as a weapon to fight viruses. PROTACs can induce the degradation of viral proteins and host cell proteins. The application of PROTACs as degraders of the hepatitis C virus protease NS3/4A was described in the study.[66] Since 2020, this strategy was investigated using targets in the SARS-CoV-2 propagation.[67] The construction of PROTAC capable of inducing the degradation of the SARS-CoV-2 spike protein was described in the study.[68] A series of PROTACs targeting prostaglandin E synthase type 2 (PGES-2), which is a host protein rather than the viral protein, were synthesized.[69] Compounds **1** and **2** of this series proved to be more effective SARS-CoV-2 replication inhibitors than the inhibitor PGES-2.[69]

Protein degraders for therapy of neurodegenerative and immune diseases were also developed;[5,70] for example, the compound QC-01-175.[5]

1, **2**

X = (CH₂)₆ (**1**), (**2**)

QC-01-175
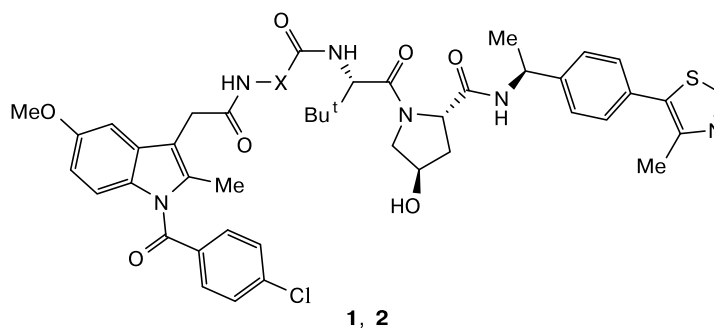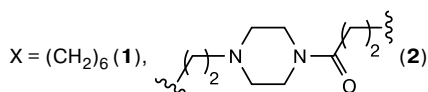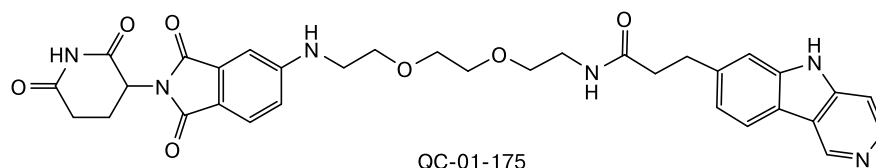
2. Advantages of PROTAC

The PROTAC technology has brought about a paradigm shift in the therapeutic effect on targets from the inhibition of biochemical (in particular, enzymatic) properties of the protein to the protein degradation. This technology has the following important advantages over classical pharmacologically active compounds.

First, the big challenge is the development of resistance of malignant tumors to chemotherapy, both cytotoxic and targeted.[71,72] Thus, first successful practical applications of protein kinase inhibitors were followed by the development of drug resistance and disease progression.[73] Antibodies blocking the immune checkpoints of PD-1 (*programmed cell death 1*)[74] and CTLA-4 (*cytotoxic T-lymphocyte-associated protein 4*)[75] were used in order to overcome drug resistance. However, antibodies recognize proteins on the plasmatic membrane, which limits their therapeutic potential. Besides, the immunotherapy resistance was observed.[76] Unlike enzyme inhibitors, PROTACs do not occupy catalytic or other sites of a protein but induce its degradation and removal from the cell. This mechanism may play a significant role in overcoming drug resistance or preventing the emergence of acquired resistance.[77] Due to the low molecular weight, PROTACs can be targeted to intracellular proteins, as opposed to antibodies.

Second, the therapy with traditional small-molecule inhibitors is based on the interaction with the active site of the target protein. This limits the application of inhibitors to numerous nonenzymatic proteins, such as transcription factors and cytoskeletal proteins.[78] For example, focal adhesion kinase (FAK) not only has an enzymatic function but functions as a scaffold for some signaling proteins. Therefore, FAK plays a significant role in the regulation of the tumor invasion, angiogenesis, and metastasis. Attempts to inhibit the FAK activity by blocking the catalytic site failed, because this approach affected only one function.[79] Meanwhile, PROTACs aim to induce proteolytic degradation of the target, thereby eliminating both the enzymatic and nonenzymatic functions. A FAK degrader, compound **3**, synthesized in the study[5] proved to be highly efficient in the inactivation of FAK and the inhibition of FAK-mediated migration and cell invasion.

Therapeutically important enzymes were found to have specific tertiary-structure elements (grooves, pockets) involved in interactions with low-molecular-weight compounds.[80] Meanwhile, the specific features of these structures may be responsible for a low inhibitor binding affinity to the target, thereby interfering with enzyme inactivation. Traditional catalytic inhibitors are ineffective against nonenzymatic proteins, while PROTACs can degrade proteins previously considered undruggable, such as FRS2α,[81] BAF,[82] the transcription factor STAT3,[83] and KRAS G12C.[84]

Third, a high drug concentration is generally required to maintain a high level of inhibition *in vivo*, which increases the risk of undesirable off-target effects. On the contrary, PROTACs act as catalysts: the ubiquitination of the target protein can be followed by the dissociation of the ternary complex, which allows the chimeric compound to be involved in new cycles of proteolytic degradation. This makes it possible to use low doses of the compound and decrease the general resorptive effect.[85]

3. Chemical structures of PROTACs

The design of PROTAC molecules with desired properties and activity is a labor-intensive and often empirical process.[86] In the preliminary PROTAC de-

3
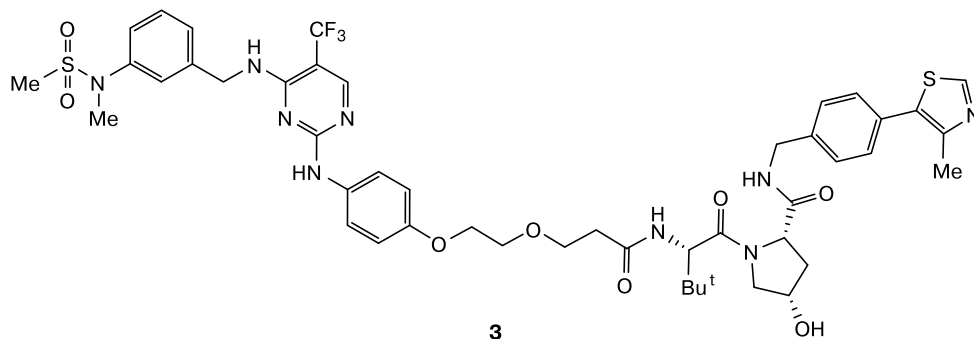
sign, attention is given to both the molecule as a whole and its components. Initially, PROTACs were developed based on known ligands for proteins, such as hormone receptors,[87] BET family proteins,[88,89] and protein kinases.[90—94] In recent years, the area of application of PROTACs was expanded, particularly due to the targeting of proteins or protein complexes previously considered undruggable: FRS2α,[81] SMARCA2,[95] Tau.[96] Therefore, the discovery of new chemical ligands for proteins is an important issue to be addressed in order to optimize the PROTAC technology.

The range of the used E3 ligase ligands was extended. As mentioned in Section 1, the early studies focused only on CRBN and VHL ligases, while more recent studies concerned with interactions with other E3 ligases, *e.g.*, MDM2,[97] IAP,[98] RNF4,[46] AhR,[99] RNF114,[49] and other representatives of the RING family. The human genome encodes more than E3 ligases. Appropriate ligands for ubiquitin ligases of the U-box, HECT, and RBR families are unknown.

The discovery of tissue-specific E3 ligase ligands is promising for the enhancement of the selectivity of PROTACs.[100] For example, phase II clinical trials demonstrated that navitoclax (ABT-263), acting as a BCL-X_L/BCL-2 ligase inhibitor, has a non-target toxicity against human platelets. The PROTAC DT2216 recruiting the VHL ubiquitin ligase was designed based on navitoclax.[101] The protein VHL is weakly expressed in platelets. Therefore, the degrader DT2216 showed not only higher efficacy against cancer cells but also lower toxicity against platelets. The compound DT2216 is in phase I clinical trials (NCT04886622).[61]

The third component of PROTACs is a linker between functional groups, which also plays an important role in the chimera design. The linker dictates the distance between the target protein and the ubiquitin ligase, which directly affects the efficacy of the degrader. To target FMS-like tyrosine kinase 3 (FLT3) with the mutation known as internal tandem duplication (ITD), a small library of PROTACs with dovitinib as the target protein ligand and 17 linkers of different length was synthesized.[102] Only compounds **4** and **5** exhibited antiproliferative activity against the leukemia cell lines MOLM-13 and MV-4-11.

Generally, several chimeras with linkers of different length or with different chemical composition are synthesized. Alkyl, alkylated triazole (compound **6**), and PEG chains (compound **7**) and compounds containing acetylenic moieties (ARD-69) or piperazine and/or piperidine rings (compounds **8**, ARD-69, ARV-471) are most often used as such linkers.[103] This enables one to compose different ternary complexes consisting of POI, E3, and PROTAC and find the most successful candidate compound by comparing the efficiency of these complexes. It is difficult to predict which combinations of an E3 ligase ligand, a linker, and a target protein ligand will be the optimal one. Therefore, the development of new approaches to the rational design of linkers is another research area related to TPD.

Since there are numerous linkers and functional components, there is a need to digitalize the PROTAC design. The web-based open-access database (http://cadd.zju.edu.cn/protacdb/), which integrates the structural information and experimental data for PROTACs, was developed for this purpose.[104] Currently, the PROTAC-DB consists of 2258 PROTACs, 275 protein ligands, 68 E3 ligase ligands, and 1099 linkers, as well as their chemical structures, biological activities, and physicochemical properties.

The efficacy of PROTACs directly depends on the formation of the POI—PROTAC—E3 ligase ternary complex. The first crystal structure of the complex of the PROTAC MZ1 with VHL and the BRD4[BD2] target was determined in 2017.[105] This study revealed numerous protein—protein interactions between VHL and BRD4[BD2] formed *de novo*. Subsequently, the crystal structures of other PROTAC ternary complexes were established.[106,107] These data confirm that an increase in the efficiency of degraders is based on the formation of protein—protein interactions between the E3 ligase and POI. The formation of a ternary complex may be more important than the ligand binding affinity to the

4, 5

$n = 4$ (**4**), 5 (**5**)

6

7 (*n* = 1, 2)

8 (*n* = 2—18)
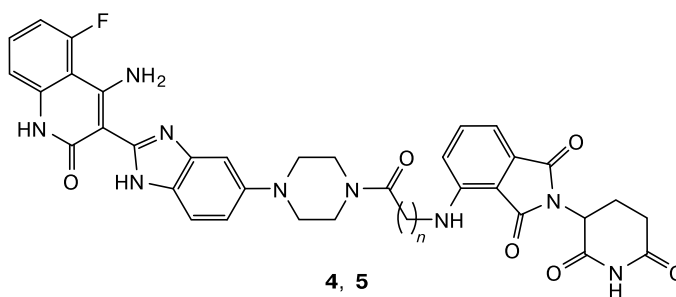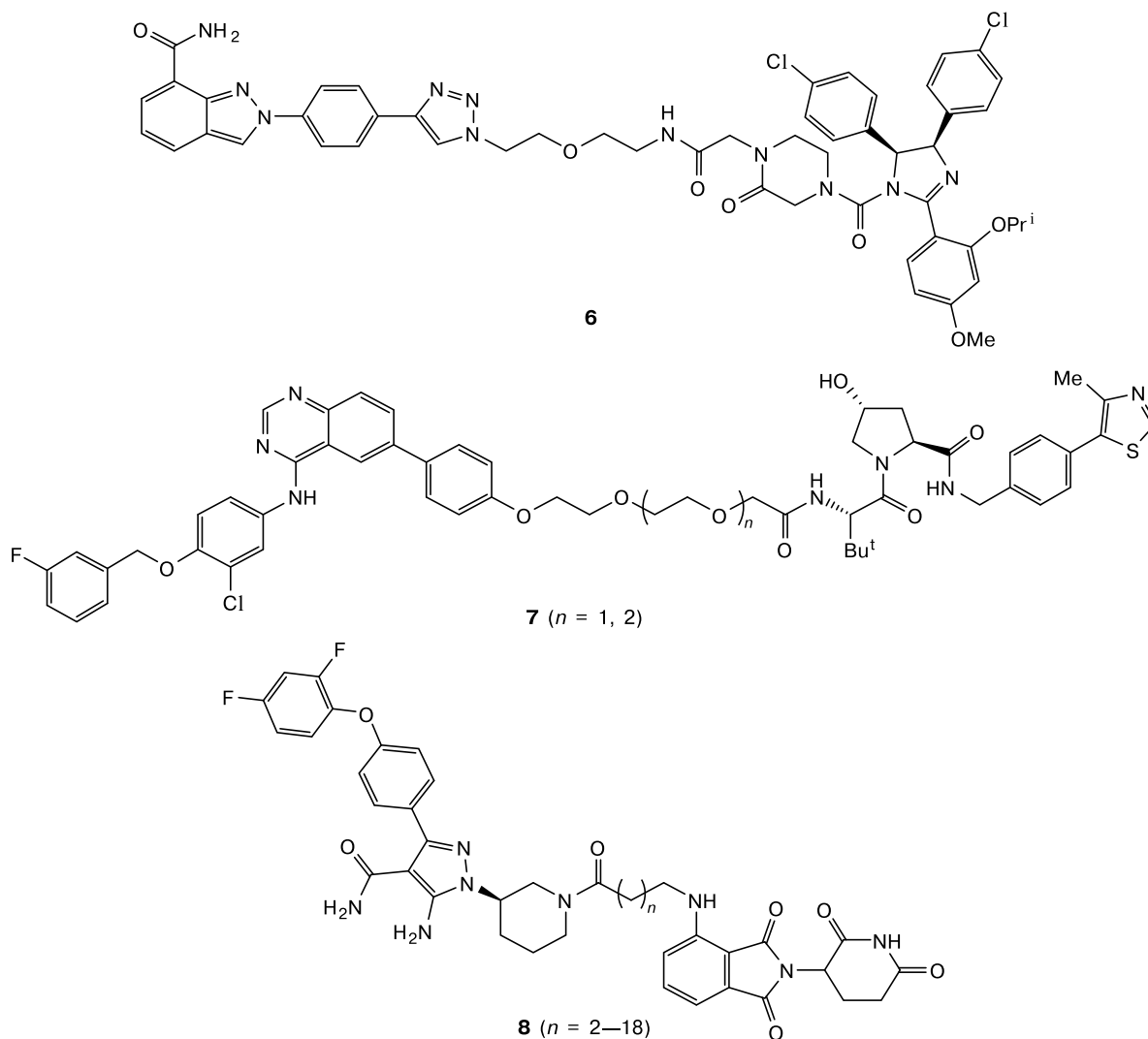
target. In the study,[91] it was demonstrated that the protein p38a/MAPK14 is efficiently degraded in the case of a low ligand binding affinity to the target protein, but with the formation of a ternary complex.[91]

Interestingly, this property is yet another advantage of PROTACs over enzyme inhibitors. A number of studies demonstrated that some PROTACs can be selective for isoforms or subtypes of targets, even if the selectivity was not initially expected. For example, PROTACs were developed that are selective for HER2 but not for EGFR;[91] selective for CDK4 over CDK6;[94] selective for STAT3 over other isoforms of STAT,[108] and selective for BCL-X$_L$ over BCL-2,[101] although the ligands used in the PROTACs were not selective for the corresponding targets. Apparently, the ability of these PROTACs to induce the isoform-specific degradation is due to the preference for ternary complex formation with one particular POI isoform over others.[109] It is

worth noting that the efficiency of the POI degradation depends also on other factors, such as the stability of PROTAC and the intensity ratio of the biosynthesis and catabolism (steady-state level) of POI and E3 ligase in the cells.

In silico approaches are used to predict the ternary complex formation and simulate interactions of PROTACs with the target protein and the E3 ligase.[110] The Rosetta program predicted active PROTACs based on the published structures of active molecules.[111] Recently, this program was improved and renamed to PRosettaC (derived from PROTAC) (https://prosettac. weizmann.ac.il/pacb/steps).[112] A model of the BTK/ CRBN complex was rather accurately predicted using the PRosettaC program based on the experimental results with several PROTACs. It would be expected that the computational modeling will be useful in the rational design of chimeras for TPD.

4. PROTACs in cancer therapy

Targeted cancer therapy is based on the selective action generally *via* intracellular signaling pathways in cancer cells. Small-molecule signaling inhibitors have limitations (see Section 2). Nevertheless, such functional inhibitors provided the basis for the development of anticancer PROTACs. Important targets for cancer therapy using PROTACs and the state-of-the-art patents are analyzed below.

4.1. Nuclear hormone receptors

Androgen receptor (AR). Interactions of the androgen receptor (AR) with androgens as physiological ligands are responsible for human prostate epithelial cell proliferation. The disruption of AR-mediated signaling provides the basis of the targeted prostate cancer therapy.[113] The long-term use of AR antagonists, *e.g.*, enzalutamide, an androgen receptor inhibitor, which suppresses the translocation of hormone-activated receptors to the nucleus and binding to DNA, leads to the rescue of cancer cells from the antiproliferative effect of AR antagonists and disease progression.[114]


Enzalutamide
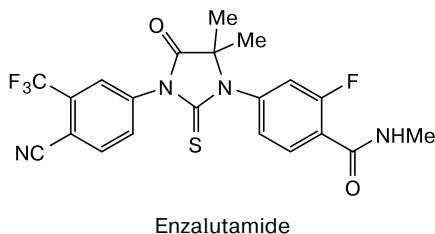
To overcome this problem, the highly active AR PROTAC ARD-69 recruiting VHL was synthesized.[58] This compound induced pronounced AR degradation in AR-positive prostate cancer cell lines and efficiently reduced levels of the AR protein and prostate-specific antigen in xenograft models.[58] The compound ARD-61, a close analog of ARD-69, recruiting VHL was devel-

oped more recently.[115] The compound ARD-61, the most potent AR degrader, inhibits the tumor growth *in vivo* in models of castration-resistant prostate cancer and AR-positive breast cancer.[115,116] Therefore, the AR degradation can eliminate the AR function, which cannot be achieved using enzalutamide.

Bavdegalutamide ARV-110 developed by Arvinas was the first PROTAC, which showed encouraging results in clinical trials.[117] This oral compound induced the degradation of mutant AR and had a satisfactory safety profile (NCT03888612).[118] One confirmed partial RECIST (response evaluation criteria in solid tumors) response was seen in a patient with a prostate-specific antigen level reduced by 97% and a tumor size decreased by 80% against the background of the resistance to enzalutamide, abiraterone, and bicalutamide.[117] The compound ARV-110 was included in the patent applications US20180099940 and US2021113557 and later in the worldwide patent application WO2021231174.

The compound CC-94676 (AR-LDD), an AR PROTAC, which was initially developed by Celgene and later by Bristol Myers Squibb (BMS),[119] showed activity similar to that of ARV-110 in preclinical trials. It efficiently degraded AR, had favorable pharmacokinetic properties, and induced sustained suppression of tumor growth in mouse models. The compound CC-94676 is in phase I clinical trials (NCT04428788). Meanwhile, the compounds CC-94676 and ARV-110 were not directly compared in preclinical or clinical trials. The structure of CC-94676 was not published in open access.

Estrogen receptor alpha (ERα). The estrogen receptor regulates the gene expression in the breast epithelium. Compounds having an ER-mediated effect, from progestins and the ER modulator tamoxifen to fulvestrant as a selective receptor antagonist, showed significant clinical effectiveness in managing breast cancer. However, many patients acquired ER inhibitor resistance.[120] The oral PROTAC ARV-471 was developed by Arvinas for


ARD-61

Fulvestrant

Palbociclib
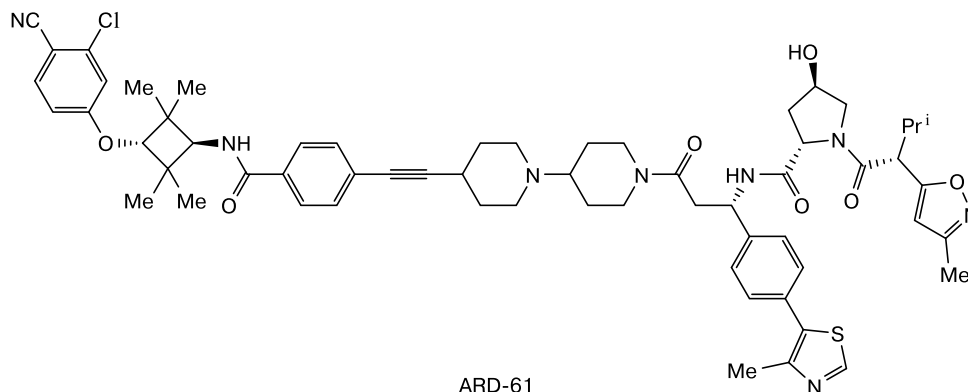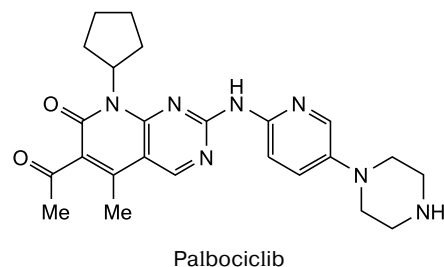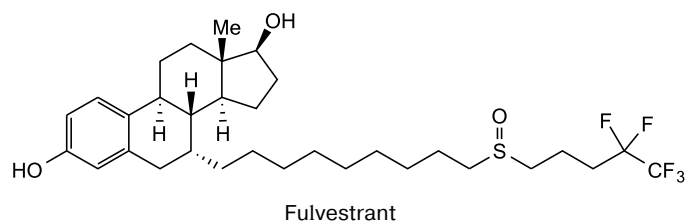
the ER degradation. In preclinical trials, the compound ARV-471 induced the degradation of ERα in a number of ER-positive breast cancer cell lines ($DC_{50} = 0.9$ nmol L^{-1} in the MCF7 cell line). The compound ARV-471 has a higher *in vivo* activity compared to fulvestrant, which is enhanced in combination with palbociclib, a cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitor.[121] The compound ARV-471 was the second PROTAC after ARV-110 that entered in clinical trials (NCT04072952). The compound ARV-471 was disclosed in the patent application WO2018102725. Compared to other selective ER degraders in early clinical trials, the compound ARV-471 showed the highest efficacy in ER degradation with the lowest adverse effects. Currently, ARV-471 is in phase II clinical trials; its combination with palbociclib, in phase Ib.[119]

4.2. Protein kinases

Bruton's tyrosine kinase (BTK). This non-receptor tyrosine kinase plays a key role in the maturation and function of B-cells and immune responses. Bruton's tyrosine kinase inhibitors (*e.g.*, ibrutinib)[122] were approved by the FDA to treat B-cell malignancies, such as chronic lymphocytic leukemia and mantle cell lymphoma (MCL). However, in many patients, drug resistance is acquired mainly by BTK^{C481S} missense mutations.

In the studies,[123,124] the PROTACs P13I and L18I were developed based on ibrutinib and the CRBN ligand. These compounds induced the degradation of wild-type BTK and C481S mutants and, as a consequence, the suppression of cell growth of diffuse large B-cell lymphoma and MCL.

Ibrutinib
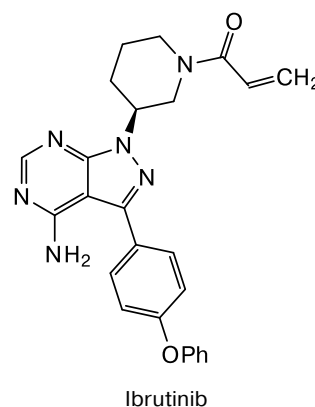
However, the compounds P13I and L18I form covalent bonds with BTK, which interfere with the detachment of the PROTAC and new cycles of proteolysis. Hence, the degraders RC-1[125] and RC-3[126] were developed, which form reversible covalent bonds with BTK. This results in the enhancement of the efficacy and selectivity of PROTACs. Besides, the new generation non-covalent BTK inhibitors RN486[127] and CGI1746[128] were used to construct PROTACs targeting BTK.

Cyclin-dependent protein kinases 4 and 6 (CDK4/6) phosphorylate the retinoblastoma protein (Rb), activating the transcription factor E2F and cell proliferation.[129] An increased CDK4/6 level in tumors allows one to consider these kinases as therapeutic targets. In the study,[130] pomalidomide (CRBN ligand), palbociclib (CDK4/6 inhibitor), and a 1,2,3-triazole linker were exploited to synthesize the PROTAC pal-pom. The chimeric structure of pal-pom induced the degradation of CDK4/6 with DC_{50} of 20—50 nmol L^{-1} and the cell cycle arrest in triple-negative breast cancer cells.

P13I (X = C=O),
L18I (X = CH$_2$)

RC-1

RC-3

CGI1746

RN-486

pal-pom
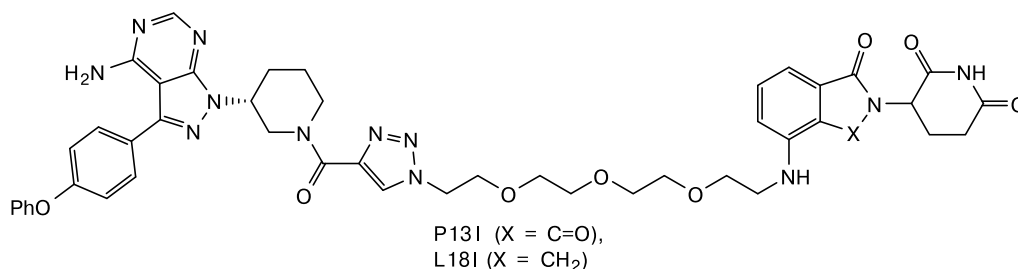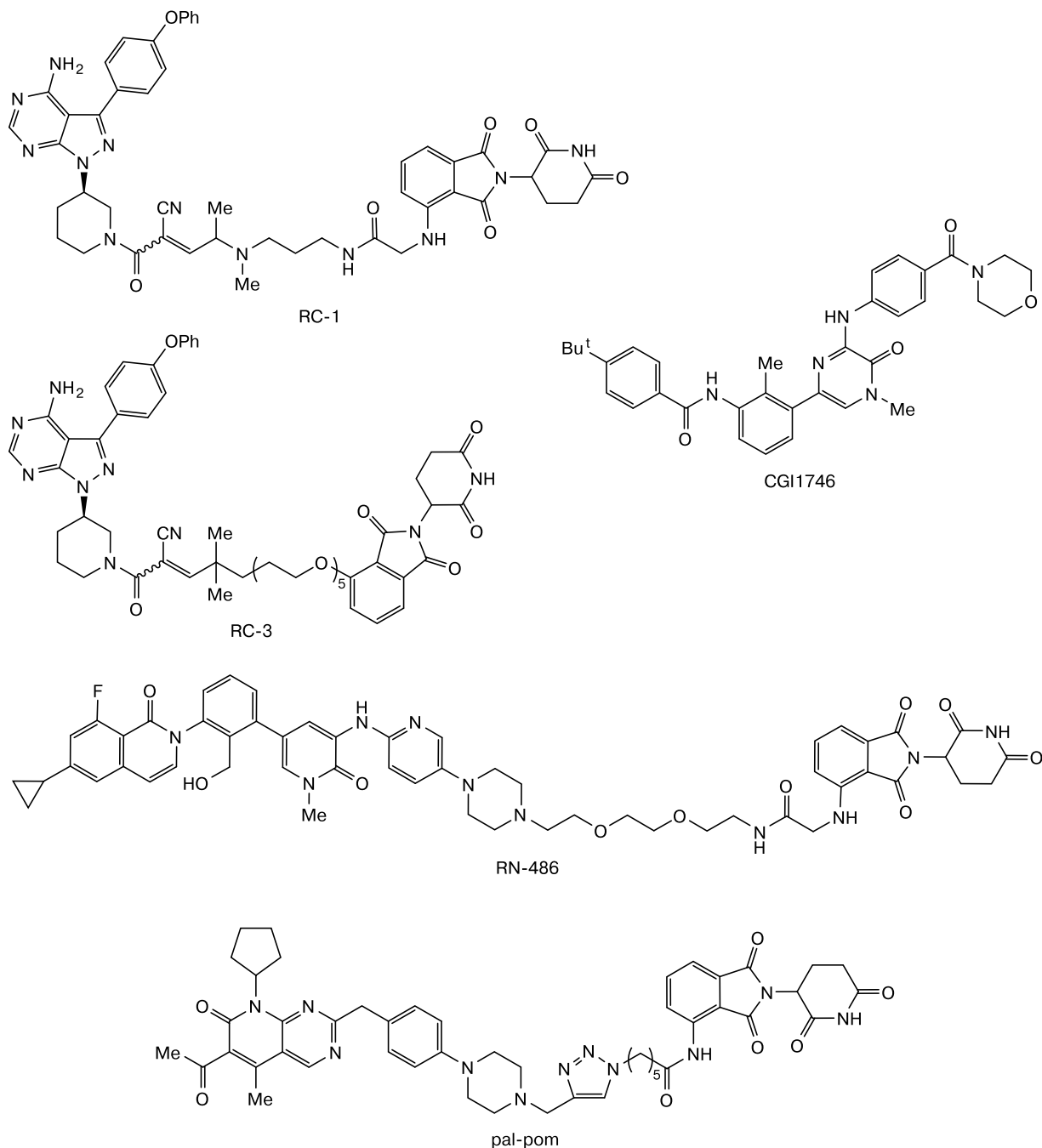
A high affinity of the amino acid sequences of CDK4 and CDK6 in proximity to their active sites hinders their differentiation by inhibitors, whereas PROTACs are selective. The linkers for the CDK6-selective degrader BSJ-03-123 based on palbociclib[131] and the CDK4-selective degrader BSJ-04-132 based on ribociclib[132] were optimized. As mentioned above, the selectivity can be attributed to high efficiency of the CDK—CRBN interaction. It should be emphasized that a simple modification of the linker by introducing a glycine moiety allowed the synthesis of the new

PROTACs BSJ-02-162 (at the time of publication of the review, the structure of the compound BSJ-02-162 was not published in open sources), CP-10, and YX-2-107 selective for CDK6.[132,133]

Interestingly, CDK6 exhibits both kinase and non-kinase functions, the latter playing an important role in the pathogenesis of Ph-positive acute lymphoblastic leukemia (Ph+ALL). The PROTAC YX-2-107 inhibits the non-kinase function of CDK6, thereby more effectively suppressing Ph+ALL cells compared to palbociclib (CDK6 kinase inhibitor).[133]

BSJ-03-123

BSJ-04-132

YX-2-107

CP-10

Imatinib

Dasatinib

Ponatinib

DAS-6-2-2-6-CRBN

ABL001

GMB-805

GNF-5

GMB-5
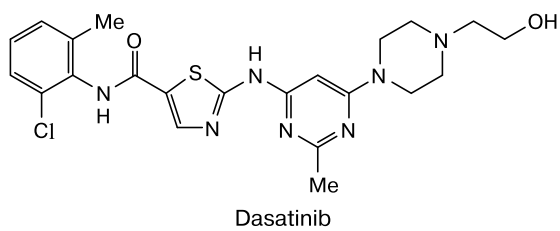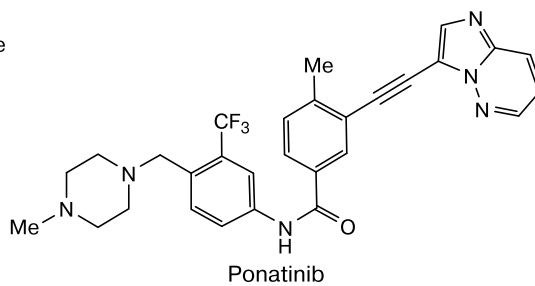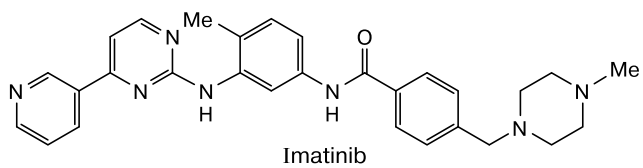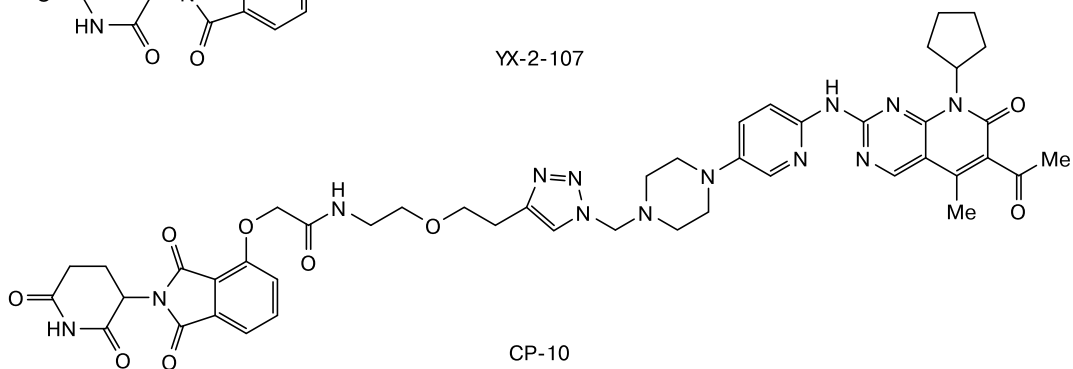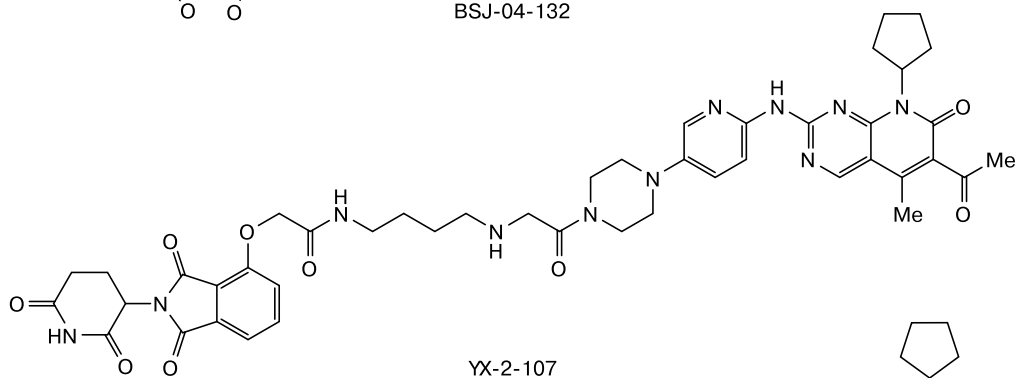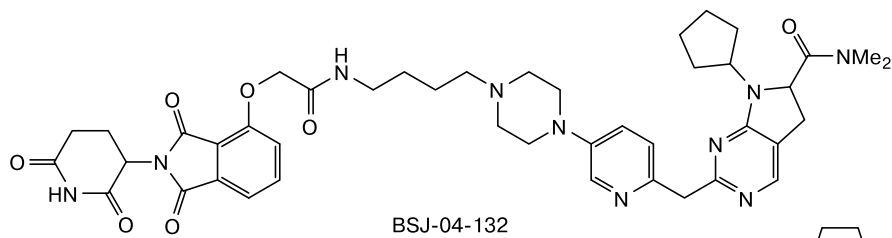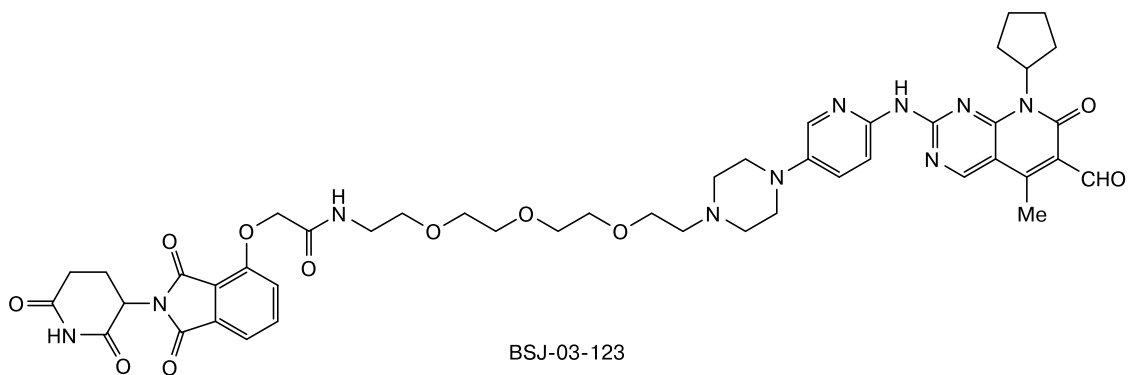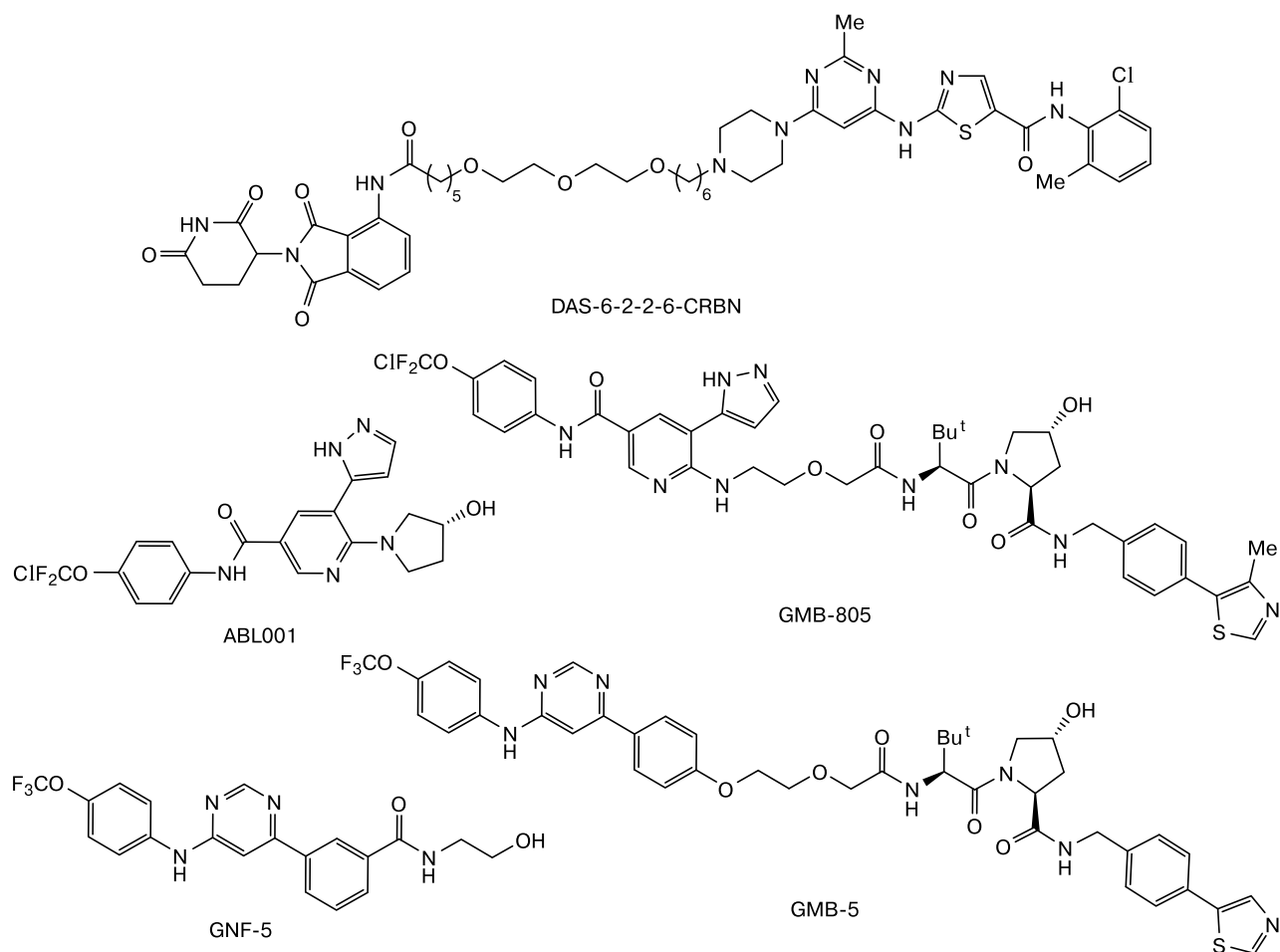
Chimeric tyrosine kinase BCR-ABL is a validated therapeutic target in chronic myelogenous leukemia (CML).[134] Although the BCR-ABL inhibitors of three generations, imatinib, ponatinib, and dasatinib, are successfully applied in the therapy of CML, patients need constant (often lifelong) treatment with the inhibitors.[135]

The drug resistance can be caused by mutations in BCR-ABL.[136] The compound DAS-6-2-2-6-CRBN, which was synthesized based on dasatinib and the CRBN ligand,[135] induced pronounced degradation of BCR-ABL and suppressed CML cell proliferation.

Effective dasatinib-based PROTACs were synthesized using ligands for the E3 ligases VHL, RNF114, and IAP[137−139] (Fig. 4).

The active and selective PROTACs GMB-805 and GMB-5 having lower adverse effects were developed based on ponatinib and the new BCR-ABL inhibitors ABL001 and GNF5, respectively.[140−142]

Fig. 4. Fragments of the inhibitors of the E3 ligases VHL (*a*), RNF-114 (*b*), and IAP (*c*) in dasatinib-based PROTAC structures.

BI-4464

PF-562271
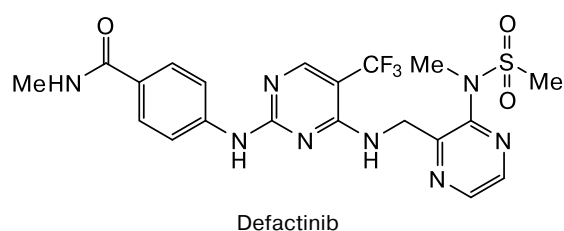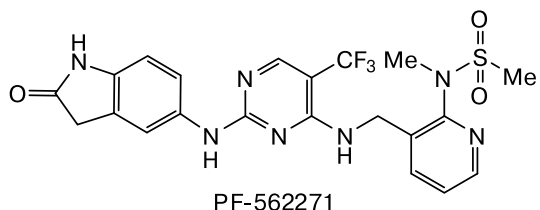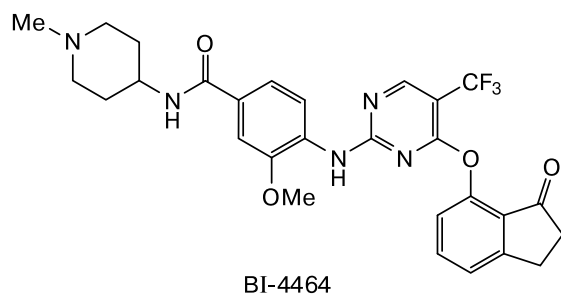
Defactinib

Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase that regulates the tumor invasion and metastasis.[143] The overexpression of FAK is correlated with an unfavorable clinical outcome. Hence, the FAK inhibitors defactinib, BI-4464, and PF-562271 were developed.[144]
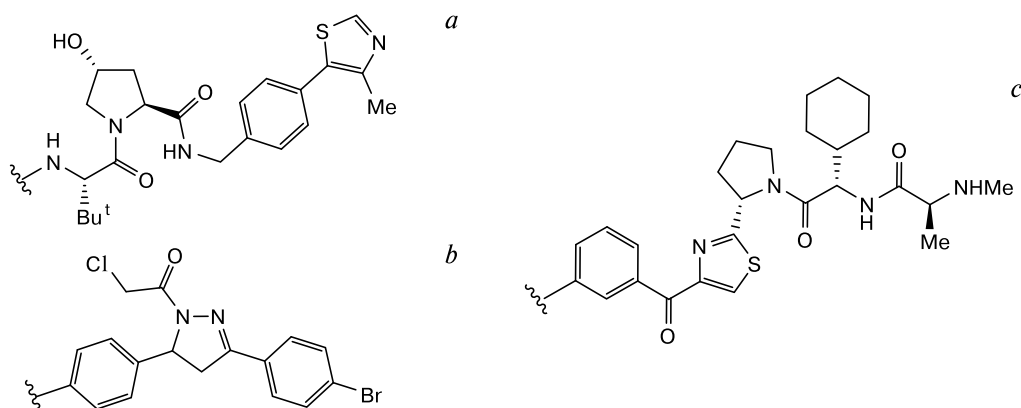
The FAK degrader PROTAC-3 was synthesized based on defactinib and VHL.[79] The binding activity was assessed over a panel of >400 protein kinases, and PROTAC-3 proved to be highly selective, because

it binds less than 20 kinases, whereas defactinib inhibits about 100 kinases. The compound PROTAC-3 reduces the migratory properties of cancer cells due to FAK degradation, whereas no significant effect was observed for defactinib. Therefore, FAK degraders, as opposed to FAK inhibitors, can act on both enzymatic and nonenzymatic functions of the protein, which significantly impairs cancer cell migration.

The PROTAC FC-11, developed based on the FAK inhibitor PF562271 and pomalidomide, induced rapid FAK degradation at picomolar concentrations.[145]

Epidermal growth factor receptor (EGFR) regulates the epithelial cell proliferation, invasion, metastasis, and apoptosis.[146] The *EGFR* gene amplification and/or the protein overexpression play an important role in the pathogenesis of esophageal cancer, glioblastoma, breast cancer, and non-small cell lung cancer (NSCLC).[147] The EGFR inhibitors gefitinib, lapatinib, and afatinib were approved for therapy, but they cause drug resistance due probably to mutations in the targets (*e.g.*, EGFR^{L858R}, EGFR^{T790M}, or EGFR^{C797S}).[148]

Series of PROTACs 1, 3, and 4 were synthesized based on lapatinib, gefitinib, and afatinib, respectively, by connecting fragments of the EGFR inhibitors to a VHL ligand by an appropriate linker. The synthesized degraders showed high antiproliferative activity in breast and lung cancer cell lines.[94] It is worth noting that this type of PROTACs is selective for different EGFRs. Thus, PROTAC 1 (compound **7**, *n* = 1) is a degrader of wild-type EGFR or the exon 20 insertion mutant of EGFR; PROTAC 3 is capable of degrading the exon 19 deletion mutant of EGFR or L858R EGFR; PROTAC 4 is a degrader of the double mutant L858R/

PROTAC-3

FC-11

Afatinib

Gefitinib

Lapatinib

dBET1
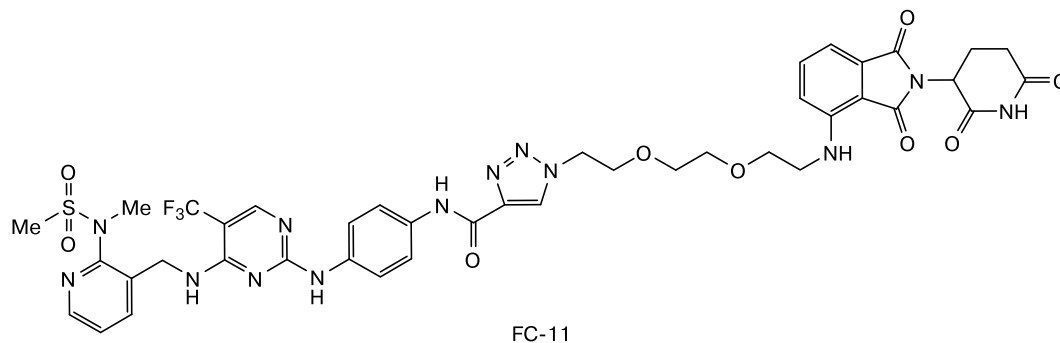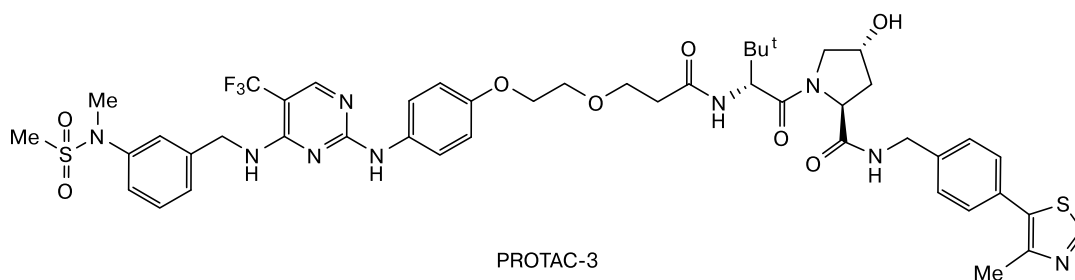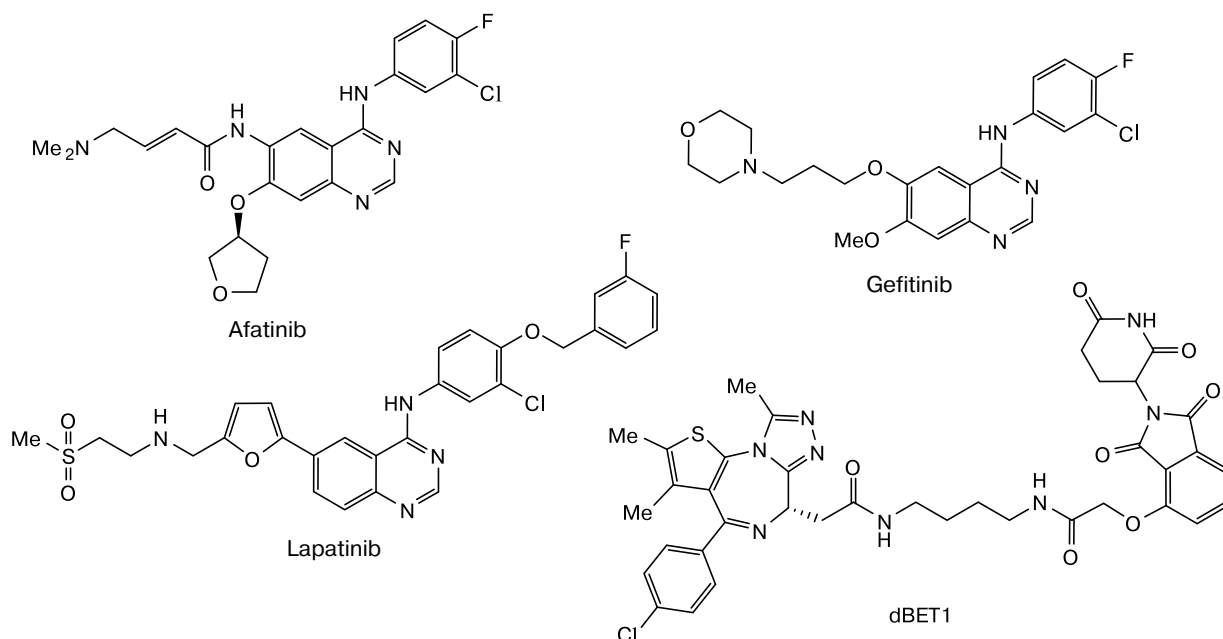
T790M EGFR. Since the selectivity of PROTACs based on EGFR inhibitors corresponds to the selectivity of the starting inhibitors,[148–152] it is important to perform molecular typing of EGFRs before using the corresponding PROTAC.

4.3. Epigenetic regulation

Epigenetic proteins play an important role in gene expression regulation. After the elucidation of the relationship between the disruption of epigenetic regulation and tumor pathogenesis, researchers focused on the development of small-molecule inhibitors targeting epigenetic enzymes.[153,154] These targets have a number of specific features, significantly limiting the therapeutic application of inhibitors.

1) Many epigenetic proteins, particularly their catalytic domains or protein—protein interaction re-

gions, are highly homologous and conserved,[155] thus making the development of selective inhibitors challenging. Examples are the bromodomain and extraterminal domain (BET) family and the catalytic domains of histone deacetylases (HDAC).

2) Generally, epigenetic proteins consist of several domains and are involved in multisubunit complexes. Therefore, the blockade of one functional part or one protein of the complex not necessarily leads to the desired result, because other activities will remain unchanged.[156]

The screening using RNA interference and the genome editing demonstrated the therapeutic importance of completely removing specific proteins. Therefore, TPD mediated by PROTAC technology is the optimal strategy for epigenetic targets. This approach not only makes it possible to avoid or slow down the development and spread of drug resistance but also offers opportunities unavailable with inhibitors.

ARV-825

QCA570
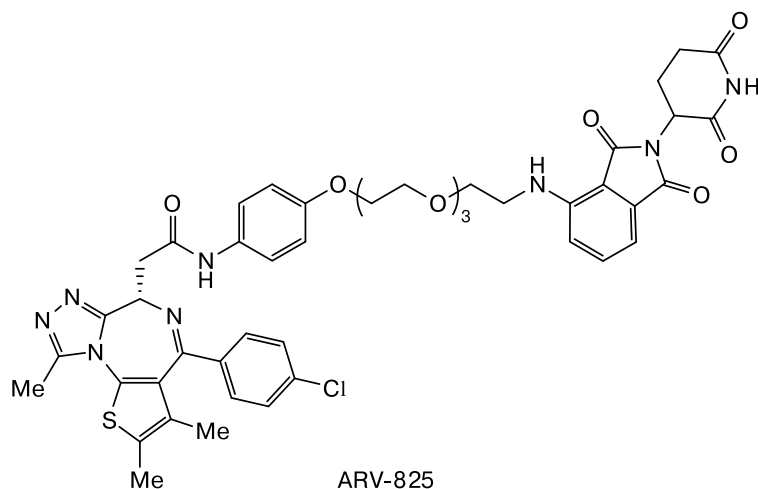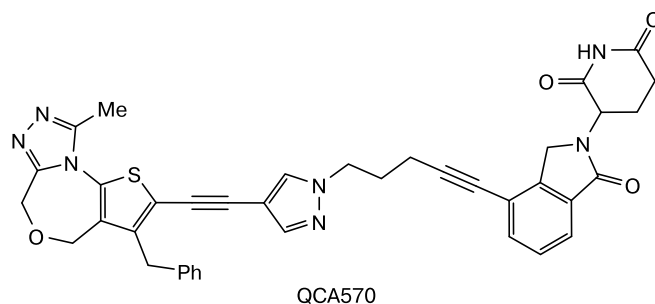
First, in the case of multidomain proteins, it is possible to selectively target the domain most easily accessible for ligation, even if this region is not functional.[157] Second, a subunit of the multiprotein complex most susceptible to proteolysis can be used to remove other subunits of the complex, which could previously not be targeted.[157,158]

BRD4, a representative of the BET protein family, is a reader of histone acetylation, triggering the gene transcription, the products of which activate proliferation (in particular, the *c-Myc* oncogene).[159] The PROTAC ARV-825, which induced the proteolysis of BRD4, was developed based on pomalidomide (CRBN ligand) and OTX015 (BRD4 inhibitor).[160] Compared to OTX015, ARV-825 showed higher inhibitory activity against the BRD4 transcriptional targets c-Myc, CDK4/6, JAK2, STAT3/5, *etc.* with increasing expression of p21 and p27.

The compound dBET1 was synthesized by connecting the BRD4 inhibitor JQ1, the CRBN ligand, and a short aliphatic chain serving as a linker.[32] At nanomolar concentrations, the compound dBET1 induced the degradation of BRD4 and significantly reduced the levels of the proteins c-Myc, BRD2, BRD3, and BRD4. The compound dBET1 proved to be more effective in leukemia xenograft models compared to JQ1. It is worth noting that dBET1 and ARV-825 contain the same BRD4 and CRBN ligands, but the differences in the activity of the degraders is achieved due to a change in the structure of the linker. Thus, ARV-825, which is a significantly more effective degrader than dBET1, contains PEG as the linker.

The PROTAC QCA570, a BET degrader containing the selective BRD4 ligand (BD1) and an alkylated triazole linker, was described in the study.[161] At pico-

molar concentrations, the compound QCA570 induced degradation of the target protein and caused complete and durable tumor regression in leukemia xenograft models.

BRD9, a component of a chromatin-remodeling multiprotein complex regulating the gene expression, is important in addressing the biology of synovial sarcoma.[162] The degrader CFT8634 developed by C4 Therapeutics as a BRD9 PROTAC[94] is superior to existing BRD inhibitors due to its high specificity towards BRD9 degradation over BRD4/7. Therefore, the compound CFT8634 is likely to be less toxic than the existing BRD inhibitors. It is important that the compound CFT8634 demonstrated also excellent activity in synovial sarcoma cell lines and *in vivo*, suppressing the patient-derived xenograft tumor growth.

The compound FHD-609, a BRD9 degrader designed by Foghorn Therapeutics,[119] is currently in phase I clinical trials for patients with synovial sarcoma (NCT04965753). Apart from PROTACs targeting BRD4 and BRD9, Foghorn Therapeutics developed PROTACs for HDAC,[163–166] PCAF/GCN5,[167] SMARCA2/4,[82] and other epigenetic proteins.

4.4. Miscellaneous proteins as targets for PROTAC technology

Anti-apoptotic protein BCL-X_L of the BCL-2 family regulates mitochondrial processes during cell death. The overexpression of the protein BCL-X_L in cancer cells is a factor of drug resistance.[168] Small-molecule BCL-X_L inhibitors, for example, navitoclax, ABT-263, and A-1155463, were developed; however, side effects, in particular severe thrombocytopenia, limit their use as anticancer drugs.[169]

CFT8634
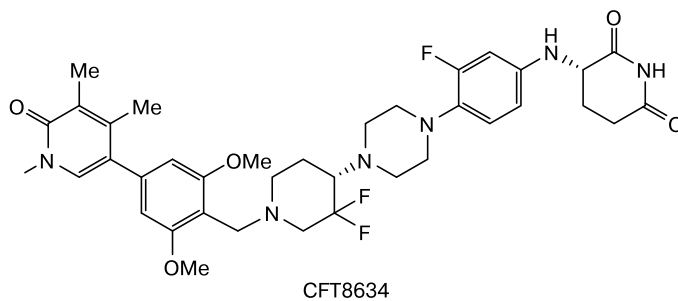
The degrader DT2216, developed by Dialectic Therapeutics, is a PROTAC, in which a navitoclax moiety is connected to the VHL ligand. The compound DT2216 is a more efficient BCL-X$_L$ degrader compared to navitoclax;[101] it did not cause thrombocytopenia because VHL is weakly expressed on platelets. Due to the promising preclinical results, DT2216 is currently in phase I clinical trials for patients with progressing or metastatic solid tumors and hemoblastosis (NCT04886622). In 2019, Bioventures filed the worldwide patent application WO2019144117 for DT2216.

Navitoclax was also utilized to develop the CRBN-recruiting PROTAC XZ739 for the treatment of T-cell acute lymphoblastic leukemia (T-ALL)[170] and the

PROTAC 8A recruiting the IAP E3 ligase for the degradation of BCL-X$_L$ in cutaneous T-cell lymphoma cells.[171] Since the expression of VHL and CRBN in this tumor is low, PROTACs containing these ligase ligands were ineffective.

Signal transducer and activator of transcription STAT3 regulates the expression of genes involved in survival, proliferation, and invasion of tumor cells, tumor angiogenesis and metastasis.[172] A considerable success in the therapeutic targeting of STAT3 was achieved due to the development of the potent selective PROTAC SD-36.[83] The compound SD-36 containing a lenalidomide analog as the CRBN ligand and the STAT3 inhibitor SI-109 induced the pronounced and

SD-36

XZ739

8A
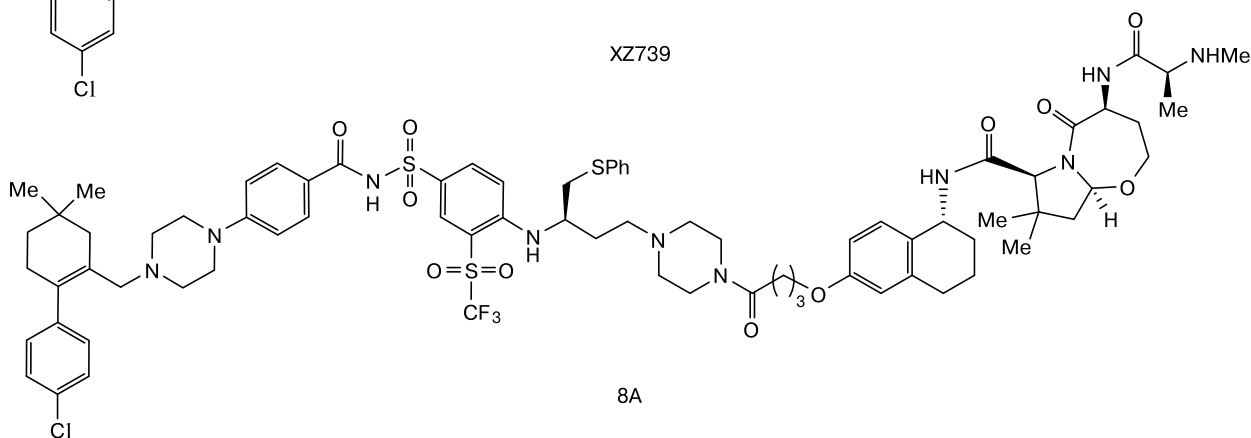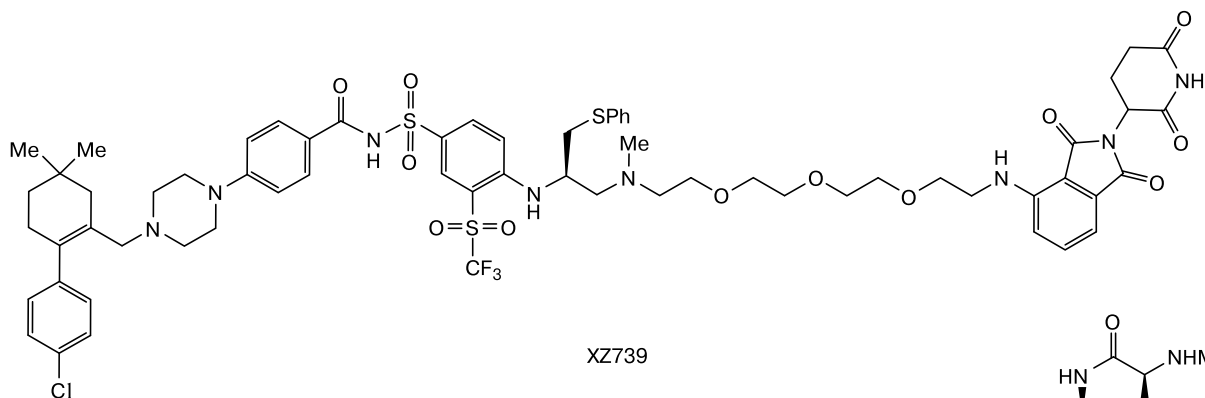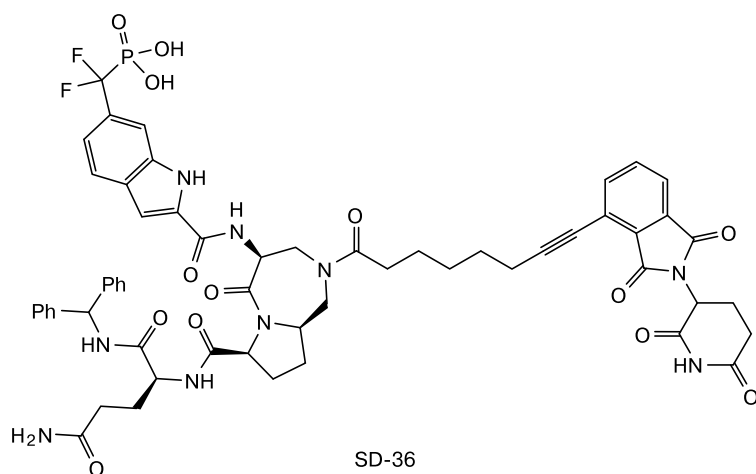
rapid degradation of STAT3 in hemoblastosis cell lines. It is worth noting that SD-36 is more selective for STAT3 over other isoforms of the STAT family, although SI-109 is not isoform-selective. The compound SD-36 induced long-lasting STAT3 degradation in mouse tumor models and complete regression in xenograft models of megakaryoblastic leukemia MOLM-16, the large-cell lymphoma SU-DHL-1, and the anaplastic large-cell lymphoma UP-M2 during two weeks after the termination of the treatment with the PROTAC.

5. Limitations of PROTAC technology

PROTACs have some drawbacks. Chimeras are more toxic than the corresponding small-molecule inhibitors.[85] Besides, ROTACs can induce non-targeted degradation of other proteins. For example, PROTACs containing immunomodulatory (IMiD) ligands for CRBN E3 ubiquitin ligase degrade not only the target but also other proteins (*e.g.*, the regulator of translation

GSPT1 or Ikaros proteins).[173] A new class of PROTACs, homo-PROTACs, containing a dimer of the E3 ligase ligand was designed. These structures provide robust interactions and autodegradation of E3 ligase. As a result, homo-PROTACs cannot affect other targets, thereby increasing the selectivity of action. Homo-PROTACs targeting VHL (compound CM11),[174] CRBN (compound **9**),[175] and MDM2 (compound **10**)[176] were reported.

PROTACs are high-molecular-weight compounds; their physicochemical properties fall outside Lipinski's rule of five.[177] Therefore, an issue to be addressed is to reveal the molecular properties of PROTACs important for the optimization of their structures. Most of the published PROTACs were evaluated only at the molecular and cellular levels, while the data on the pharmacokinetics and pharmacodynamics are scarce.[178] Besides, there are few examples of the optimized absorption, distribution, metabolism, and excretion (ADME) profiles. Therefore, there is not enough in-

CM11

9

10
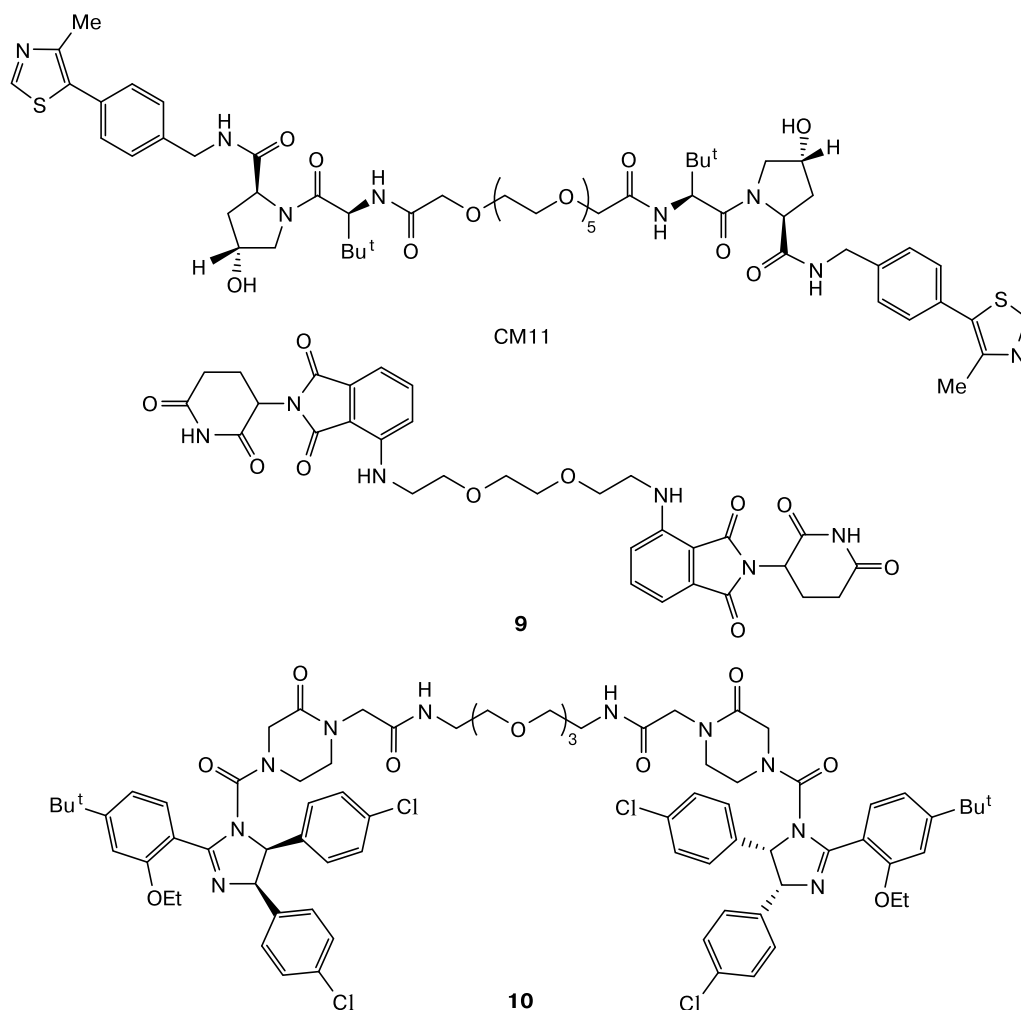
formation for the optimization of the ADME profiles to comply with the rule of five.

Heterobifunctional PROTACs are flexible and conformationally complex compounds. Therefore, the descriptors of the molecular properties (size, shape, lipophilicity, polarity, and degree of ionization), which are used to characterize small-molecule inhibitors, cannot be directly applied to the PROTAC chemical space for discovering drug candidates.[179]

The solubility and permeability are key parameters of bioavailability of PROTACs. However, the conventional tests, such as the artificial membrane permeability assay and the Caco-2 cell model, are not relevant for the evaluation of the PROTAC distribution because of issues with solubility in assay buffers and nonspecific binding.[180] The optimization and modification of these assays are required to apply them for PROTACs. It is also worth noting that among the E3 ligase ligands most widely utilized in the design of PROTACs, only CRBNs (thalidomide derivatives) are apparently suitable for oral drugs. The clinical candidates ARV-110 and ARV-471 contain CRBN ligands, whereas VHL, MDM2, and IAP ligands have higher molecular weights, a larger topological polar surface area, and higher flexibility, which are potentially unfavorable for oral drugs.[110] However, CRBN ligands have low chemical and metabolic stability because of the racemization of the glutarimide group and the hydrolysis of imide groups. This problem can be addressed by developing new E3 ligase ligands.

Conclusion

PROTACs are chimeric compounds containing a chemical moiety, which interacts with a target protein in the cell and is connected to an E3 ubiquitin ligase ligand by a linker. The targeted proteolysis provides a new promising therapy, which is particularly important if target proteins were previously considered undruggable. The progress in medicinal chemistry and molecular pathology allowed researchers to design first conjugates and evaluate their physicochemical and biological properties in cultures and animal models. Lead PROTACs were pushed to clinical trials. For the rational design of PROTACs, it is necessary to standardize the technology of the synthesis and conjugation of the moieties of the chimeric molecule, discover new target proteins, and develop procedures for targeting ubiquitin ligases and computational chemistry approaches. The PROTAC principle requires extensive investigations as a technology original for fundamental science and promising for individualized therapy.

No human or animal subjects were used in this research.

The authors declare no competing interests.

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