JSCS@tmf.bg.ac.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. *87* (7–8) 785–811 (2022)
JSCS–5558

Survey
Published 4 May 2022

SURVEY

Protein degradation induced by PROTAC molecules as an emerging drug discovery strategy

MLADEN KORAVOVIĆ[1]*, BOJAN MARKOVIĆ[2], MILENA KOVAČEVIĆ[3], MILENA RMANDIĆ[4] and GORDANA TASIĆ[1]**#

[1]University of Belgrade – Faculty of Pharmacy, Department of Organic Chemistry, Vojvode Stepe 450, 11221 Belgrade, Serbia, [2]University of Belgrade – Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Vojvode Stepe 450, 11221 Belgrade, Serbia, [3]University of Belgrade – Faculty of Pharmacy, Department of Pharmacokinetics and Clinical Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia and [4]University of Belgrade – Faculty of Pharmacy, Department of Drug Analysis, Vojvode Stepe 450, 11221 Belgrade, Serbia

(Received 9 December 2021, revised 9 March, accepted 14 March 2022)

Abstract: The traditional concept of drug discovery is based on the *occupancy*-driven pharmacology model. It implies the development of inhibitors occupying binding sites that directly affect protein functions. Therefore, proteins that do not have such binding sites are generally considered as pharmacologically intractable. Furthermore, drugs that act in this way must be administered in dosage regimens that often result in high systemic drug exposures in order to maintain sufficient protein inhibition. Thus, there is a risk of the onset of off-target binding and side effects. The landscape of drug discovery has been markedly changed since proteolysis targeting chimera (PROTAC) molecules emerged twenty years ago as a part of the event-driven pharmacology model. These are bifunctional molecules that harness the ubiquitin-proteasome system, and are composed of a ligand that binds the protein of interest (POI), a ligand that recruits E3 ubiquitin ligase (E3UL) and a linker that connects these two parts. Pharmacologically, PROTACs bring POI and E3UL into close proximity, which triggers the formation of a functional ternary complex POI–PROTAC–E3UL. This event drives polyubiquitination and subsequent POI degradation by the 26S proteasome. The development and exceptional properties of PROTAC molecules that brought them to clinical studies will be discussed in this paper.

*,** Corresponding authors. E-mail: (*)mladen.koravovic@pharmacy.bg.ac.rs; (**)gordana.tasic@pharmacy.bg.ac.rs
Serbian Chemical Society member.
https://doi.org/10.2298/JSC211209027K

(CC) 2022 SCS.

Keywords: bifunctional molecules; ternary complex; cooperativity; polyubiquitination; undruggable proteome; clinical candidates.

CONTENTS

1. INTRODUCTION
2. TRADITIONAL INHIBITORS PHARMACOLOGY
3. INDUCED PROTEIN DEGRADATION: NEW APPROACH IN DRUG DEVELOPMENT
4. PROTAC TECHNOLOGY
   4.1. Mechanistic representation of protein degradation induced by PROTACs
   4.2. The development of PROTACs throughout history
   4.3. Consideration of the physicochemical properties of PROTACs
   4.4. The influence of linkers on the physicochemical and pharmacological properties of PROTACs
   4.5. Advantages of the PROTAC -induced POI degradation over the current therapeutic modalities
5. CONCLUSIONS AND PERSPECTIVES

INTRODUCTION

The fundamental concepts embedded in drug discovery science, named medicinal chemistry, have remained mainly unchanged for the last century.[1] Since Ehrlich and Langley, when receptor pharmacology began to appear,[2] drug discovery was based on the following concepts: a) identification and optimization of molecules with decent activities on corresponding biological targets and b) finding safe and tolerable doses and dose regimens that could maintain sufficient drug concentration at the site of action in order to trigger and sustain appropriate pharmacologic effects.[1] Hence, two fundamental principles originating from pharmacology – aplenty receptor occupancy and sustained drug exposure in the target tissue – were the basis for drug discovery and optimization. Hereinafter, only inhibitors will be disclosed since they are much more present in therapy than agonists. Thus, the class of molecules that will be elucidated in the following text actually agonizes certain processes by which the inhibitory function is finally achieved.

The human proteome contains more than 30,000 proteins employed in different biological functions.[3] Taking into account that many of them are included in the onset of many different diseases, scientists attempt to manipulate them in order to achieve certain therapeutic effects. Currently, the United States Food and Drug Administration (FDA) approved drugs against about 400 human proteins.[4] More than 90 % of them are enzymes, transporters, G protein-coupled receptors (GPCRs), cluster of differentiation (CD) markers, voltage-gated ion channels and nuclear receptors.[5] These biological targets are attractive, but easily accessible to agents originating from the traditional pharmacology concept. However, it has been estimated that there are about 3,000 genes involved in disease onset. In

Available on line at www.shd.org.rs/JSCS/

(CC) 2022 SCS.

summary, the current therapies can target only 13 % (400 out of 3,000 genes) of the therapeutically relevant human proteome. Therefore, about 85 % of disease-associated proteins remain without corresponding agents that could achieve therapeutic effects.[5] To summarize, the genomic revolution found new connections between certain proteins and diseases,[6–9] but traditional drug discovery strategies are not sufficient to exploit all of these emerging chemical biology findings. There are two reasons why so many therapeutically relevant proteins are still considered as pharmacologically intractable, *i.e.*, undruggable biological targets: a) many of them do not have suitable binding sites that directly modulate their function[10] and b) some of them are intracellularly localized and as such unattainable for monoclonal antibodies (mAbs) either.[11]

2. TRADITIONAL INHIBITORS PHARMACOLOGY

Traditional inhibitors cannot always attain desirable pharmacological effects owing to their characteristics as well as attributes of the corresponding biological targets described below:

a) Biological targets for traditional inhibitors are usually enzymes and receptors containing suitable binding sites for the inhibitors, while about 75 % of the human proteome lack such binding sites. Examples of such are transcription factors, scaffolding proteins and other non-enzymatic proteins inside the cell that are unattainable for traditional medicinal chemistry.[12] The challenge of targeting such biological targets can be illustrated through the fact that very few approved agents can exploit such proteins.[13,14]

b) Medicinal chemistry strategies for the development traditional inhibitors focused on targeting specific binding sites may imply high systemic drug exposures in order to attain sufficient and sustained occupancy of biological target *in vivo.*[15] This potentially increases the risk for off-target pharmacological actions and onset of side effects.

c) The potency of traditional inhibitors depends on their affinities towards biological targets.[16]

d) Inhibition of biological targets by traditional inhibitors mainly influences catalytic functions, but not the non-catalytic ones (*e.g.*, scaffolding roles).[16]

e) Competition with overexpressed native ligand for the same biological target can occur after usage of traditional inhibitors.[16]

f) Small-molecule drugs usually disrupt the activity of one domain of multidomain scaffolding proteins, while functions of other domains and their interactions with other proteins remain preserved.[17]

g) Traditional inhibitors may induce compensatory overexpression of corresponding biological targets as well as their accumulation,[18,19] resulting in incomplete inhibition and, consequently, incomplete suppression of downstream signaling pathways.

Available on line at www.shd.org.rs/JSCS/

(CC) 2022 SCS.

h) Mutation of genes involved in pathogenesis may generate therapeutically relevant proteins containing conformational changes, which is the reason for the onset of drug resistance.[17] Examples are genes for the epidermal growth factor receptor as well as for the androgen receptor.[20,21]

All the described characteristics of traditional inhibitors and their biological targets originate from the traditional occupancy-driven pharmacology concept, which will be discussed below. Such characteristics severely impede further discovery of drugs belonging to this concept as well as achieving significant and long-term clinical benefits through their usage.[17] On the basis of all the above, it could be concluded that new modalities for targeting therapeutically relevant human proteome are required.

3. INDUCED PROTEIN DEGRADATION: NEW APPROACH IN DRUG DEVELOPMENT

Different pharmacological approaches to manipulate over therapeutically relevant proteins have emerged. Some of them are antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs) and clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9 (CRISPR-Cas9) genetic engineering technology (see Supplementary material to this paper, section S-1). Despite the emergence of new methods for affecting biological targets inside the cells, usage of small-molecule drugs will preserve their place in therapy because they are able to: a) access many organs and sites of action; b) influence multiple biological targets simultaneously; c) be produced without relatively large investments using well known development paths.[1] Hence, new medicinal chemistry strategies relying on the stated advantages of small-molecule drugs could be able to override the shortcomings of traditional pharmacology.

Protein degradation induced by small molecules is an emerging strategy which, as well as nucleic acid-based agents, has the potential to target much more proteins compared to the standard strategies focused on certain binding sites. Furthermore, strategies based on small molecule degraders maintained the pharmaceutical advantages over nucleic acid-based agents.[11] The shift in pharmacological strategy – from protein inhibition to degradation – enables affecting the proteins that have been perceived as pharmacologically intractable.[22,23] Additionally, protein degradation could act synergistically with current therapeutic regimens based on inhibitors. As mentioned above, inhibition of certain cellular pathways could trigger target protein upregulation, which ultimately leads to inhibitor insufficiency.[24,25] Therefore, induced protein degradation (IPD) reduces the number of active proteins to be inhibited, but also resists their compensatory overexpression. Furthermore, many biological targets, which are pharmacologically tractable, have some scaffolding roles that are elusive for trad-

(CC) 2022 SCS.

itional pharmacological approaches, but that contribute to resistance mechanisms.[26–32]

In order to understand IPD, it is important to elucidate two main pharmacology concepts: Occupancy-driven pharmacology (ODP) and event-driven pharmacology (EDP, Fig. 1).[11] The onset of many diseases could be related to abnormal protein functioning. This issue has traditionally been addressed using occupancy-driven pharmacology. Namely, the applied inhibitor occupies the disease-related protein, consequently blocking its function and finally achieving therapeutic effect. The longer the protein functions are blocked, the greater is the achieved therapeutic effect. As mentioned above, sustained high local concentrations of an applied inhibitor are required for a therapeutic response and this could lead to off-target binding and adverse effects.[15] An emerging and alternative concept is presented as event-driven pharmacology. In this case the ligand triggers an *event* that ultimately reduces the level of disease-causing protein.

In summary, the ODP model is based on the following postulates:[11]

a) stoichiometric activity of the applied ligand;

b) a ligand must occupy a specific binding site that affects the protein function;

c) if non-covalent inhibitor, a ligand could dissociate from its binding site, which ultimately leads to restoration of the protein functions;

d) ligand selectivity is defined only by its binding profile.

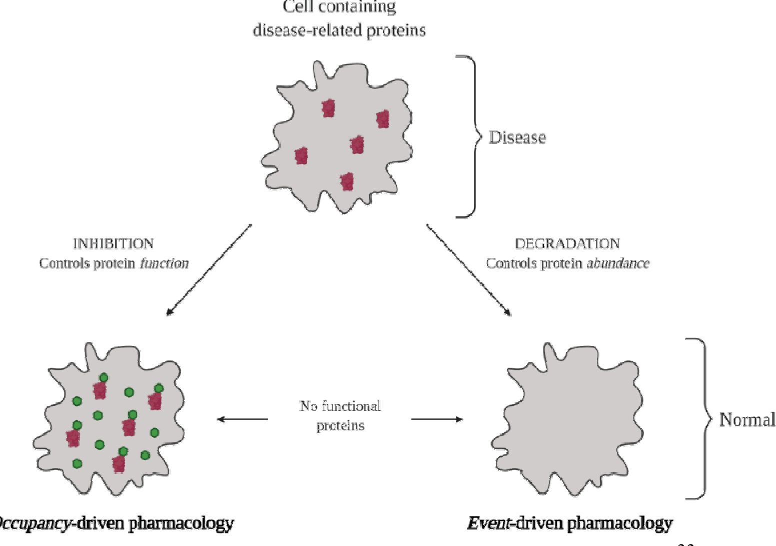

Fig. 1. Occupancy- and event-driven pharmacology models.[33]

On the other hand, the EDP model is based on more advanced postulates:[11]

a) sub-stoichiometric activity of the applied inhibitor;

Available on line at www.shd.org.rs/JSCS/

(CC) 2022 SCS.

b) a ligand does not need to occupy a specific binding site that affects protein function;

c) a ligand induce protein degradation, hence restoration of protein functions requires protein resynthesis;

d) ligand selectivity is not only defined by its binding profile.

Many modalities based on the concept of EDP have emerged, and the proteolysis targeting chimera (PROTAC) technology will be further discussed.

4. PROTAC TECHNOLOGY

Proteolysis targeting chimera technology originated from the EDP concept. This approach employs bifunctional molecules where one of their end binds the protein of interest (POI), while the other one recruits cellular quality control mechanisms, which afterwards induce protein degradation. A transient binding only of a PROTAC molecule is sufficient for its activity. In contrast to the stoichiometric occupancy of binding sites within the ODP model, PROTACs can perform multiple cycles of action and thus remove sub-stoichiometric amounts of proteins (*i.e.*, they act catalytically). Furthermore, such molecules do not need to occupy the specific binding site on the protein with high affinity in order to perform as degraders – binding at any suitable region of a biological target could potentially induce its degradation.[11] This advantage could be exploited by using different screens focused on the identification of ligands that simply bind to the biological targets without affecting their function.[34–38] Moreover, turning protein ligands into protein degraders gives an opportunity for utilizing molecules identified by high-throughput screening that were initially rejected because they simply bind to the corresponding biological targets without adequate inhibition.[39–41] As mentioned above, in comparison with the dissociation kinetics of inhibitors originating from the ODP, within the EDP the degradation of the targeted protein occurs, which ultimately requires its resynthesis. This fact gives kinetic advantage to the molecules originating from the EDP. Finally, some studies revealed that PROTACs could undergo less off-target degradation than initially suggested by the POI ligand binding profile.[42] Hence, PROTACs can provide an added layer of selectivity compared to the corresponding inhibitors.

4.1. Mechanistic representation of protein degradation induced by PROTACs

Pharmacologically speaking, PROTACs perform their biological effect (*i.e.*, protein degradation) through the active recruitment of the ubiquitin–proteasome system (UPS, see Supplementary material, Section S-2) that ultimately leads to the POI polyubiquitination and degradation by the 26S proteasome. In summary, PROTACs are bifunctional molecules consisted of a ligand for POI, a ligand for E3 ubiquitin ligase and a linker that connects the two ligands (in the following sections, ligands for POI will be in red, linkers in green and ligands for E3 ubiquitin ligases in blue, Fig. 2).[43]

(CC) 2022 SCS.

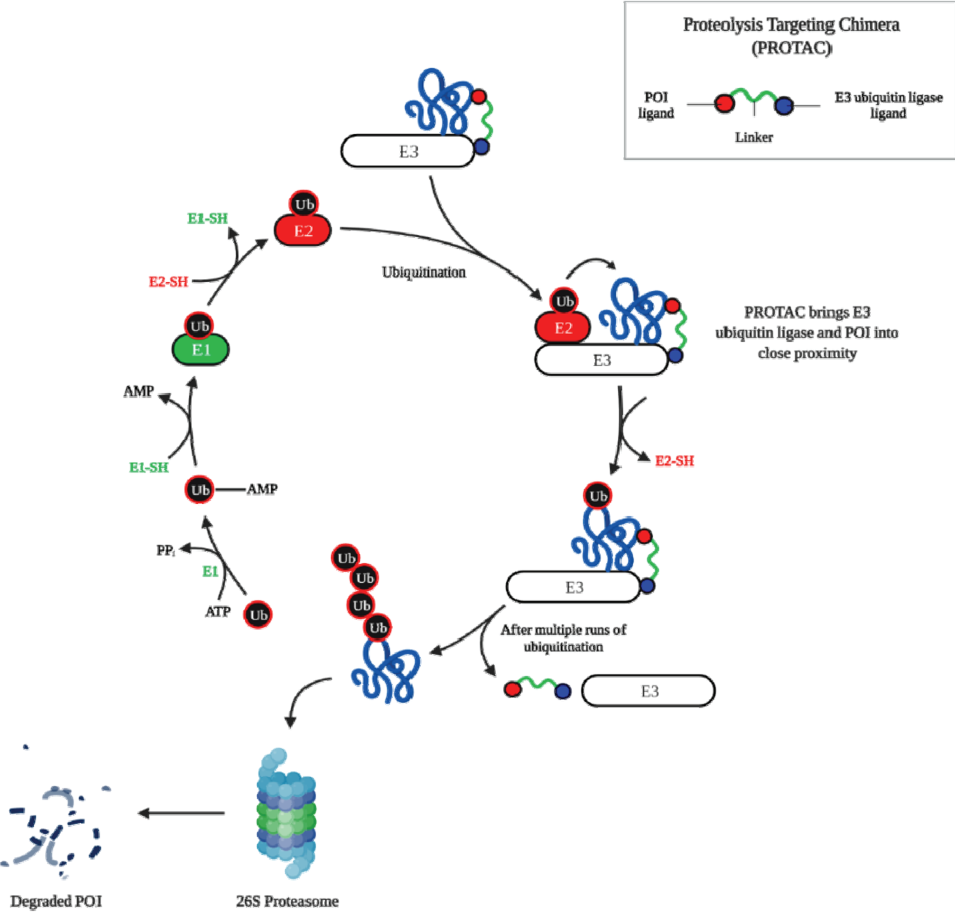

Fig. 2. General structure of PROTAC molecules and their mechanism of action.[46]

Hence, they bind POI and E3 ubiquitin ligase simultaneously which leads to proximity-induced PPIs between these two in the form of ternary complex (TC): POI-PROTAC-E3 ubiquitin ligase[44] (see Supplementary material, section S-3). The recruited E3 ubiquitin ligase mediates multiple runs of ubiquitin transfer from E2 to the POI, the TC dissociates afterwards and finally the polyubiquitinated POI is degraded in the 26S proteasome. Given that PROTAC is not degraded in this process, its destiny can be different depending on its chemical properties:[45] a) non-covalent PROTACs are able to dissociate from the TC and induce multiple cycles of degradation (catalytic mode of action (MOA)); b) if PROTAC is covalently bound to the E3 ubiquitin ligase, but non-covalently to the POI, it is still able to act catalytically (*i.e.*, undergo multiple rounds of degradation); c) PROTAC that is covalently bound to the POI cannot participate in

Available on line at www.shd.org.rs/JSCS/

(CC) 2022 SCS.

the next round of POI degradation; d) PROTACs bound in a covalent, but reversible manner to either POI or E3 ubiquitin ligase would act catalytically.

Therefore, in contrast to traditional inhibitors where continual drug binding to the biological target is necessary, PROTACs act sub-stoichiometrically and catalytically.[47]

4.2. The development of PROTACs throughout history

The first PROTACs discovered were peptide-based (Fig. 3). Although they could induce POIs degradation, their activities were in the low-micromolar range.[43]

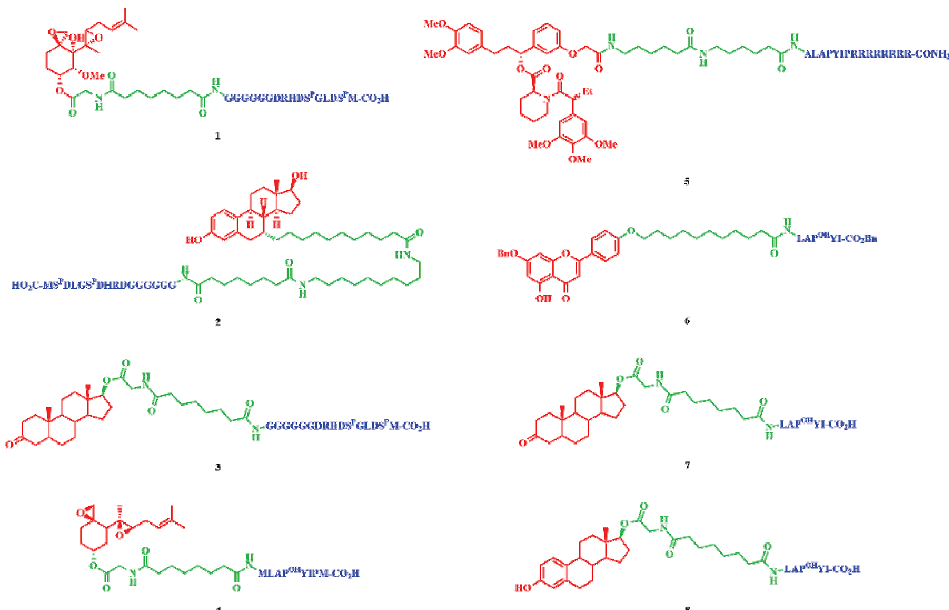

Fig. 3. Chemical structures of the first generation of peptide-based PROTACs (phosphorylated serine is denoted as "S^{P}" and hydroxyproline is denoted as "P^{OH}").

Additionally, the peptide character made these PROTACs low-permeable, which was their main shortcoming and obstruction toward further development. Ultimately, because of large molecular size, peptide-based PROTACs could potentially be recognized as antigens *in vivo*.[48]

The first PROTAC molecule (**1**, Fig. 3) was reported in 2001 by Crews and Deshaies.[49] It is a peptidic PROTAC containing a peptide ligand for the E3 ubiquitin ligase. This molecule proved the concept of selective protein degradation after their polyubiquitination. On the one hand, it consisted of IκBα phosphopeptide (IPP) as the ligand for SCF$^{\beta\text{-TRCP}}$ E3 ubiquitin ligase, while on the other hand, ovalicin as the ligand for methionine aminopeptidase-2 (MetAP-2) was

(CC) 2022 SCS.

connected by a hydrocarbon linker. In 2003, new peptidic PROTACs were reported based on the same I*κ*B*α* phosphopeptide linked to estradiol (**2**, Fig. 3) or dihydrotestosterone (**3**, Fig. 3). These PROTACs induced the degradation of estrogen receptor-*α* (ER*α*) and androgen receptor (AR), which expanded the number of degradable biological targets.[50] Degradation of AR in 293AR-GFP cells was achieved after microinjection of **3**, because the phosphate groups on the IκB*α* phosphopeptide impeded its efficient uptake into cells. Hence, this microinjection demonstrated that PROTACs can function in intact cells.[43] The first cell-permeable peptide-based PROTAC (**4**, Fig. 3) was reported in 2003.[51] It consisted of hypoxia-inducible factor-1α (HIF-1*α*) octapeptide as the ligand for VHL (von Hippel–Lindau tumor suppressor) E3 ubiquitin ligase connected *via* the linker with fumagillol as the ligand for MetAP-2. One more cell-permeable PROTAC molecule was reported one year later (**5**, Fig. 3).[52] It consisted of peptide sequence for HIF-1α connected over a linker for the FKBP12 ligand. In 2007, PROTAC molecule was reported for induced degradation of the aryl hydrocarbon receptor (AHR, **6**, Fig. 3).[53] Chemically, it has apigenin as a ligand for AHR connected through the linker for HIF-1*α* peptide. Finally, in 2008 cell-permeable PROTACs were reported that induced the degradation of AR (**7**, Fig. 3) and ER*α* (**8**, Fig. 3).[54] These molecules were composed of estradiol or dihydrotestosterone coupled through the linker with HIF-1*α* pentapeptide.

As research progressed, small-molecule PROTACs emerged in order to over overcome the above mentioned shortcomings of the peptide-based ones.[22,47,55] Significant progress in PROTAC technology was achieved by invention of small-molecule ligands for E3 ubiquitin ligases. The first PROTAC molecule based on them was synthesized in 2008 (**9**, Fig. 4).[56] The mouse double minute 2 homolog (MDM2) E3 ubiquitin ligase was recruited by nutlin, an MDM2-p53 PPI inhibitor,[57] while the other structural element was selective androgen receptor modulator (SARM). The obtained molecule was the first small molecule-based PROTAC that was able to induce the degradation of AR in the HeLa cell line after 7 h. Hence, it was proved that it is possible to make cell-permeable PROTACs, although micromolar concentrations were required to induce AR degradation.[56]

In 2008, it was found that bestatin methyl esters after binding to cellular inhibitor of apoptosis protein 1 (cIAP1) promote its autoubiquitination and degradation.[58] Hence, the Hashimoto laboratory in 2010 utilized bestatin methyl esters for synthesizing the PROTAC molecule denoted as SNIPER-2 (**10**, Fig. 5). It recruited cIAP1 as an E3 ubiquitin ligase to induce the degradation of cellular retionoic acid-binding protein (CRABP-I and CRABP-II) proteins using all-trans retinoic acid (ATRA) as the ligand for the mentioned POIs.[59] However, the synthesized PROTAC had two main shortcomings that could impede the degradation: a) SNIPER-2 induces simultaneous degradation of cIAP1 together with

Available on line at www.shd.org.rs/JSCS/
(CC) 2022 SCS.

CRABP-II, which could make POI degradation unsustainable; b) SNIPER-2 has an ester functionality prone to hydrolysis.[60] In order to overcome these drawbacks, in 2011 SNIPER-4 was invented (**11**, Fig. 5) in which the ester was replaced with an amide functionality.[60] It was found that this PROTAC induces sustained CRABP-II degradation without inducing cIAP1 degradation.

Fig. 4. Chemical structure of AR-targeting PROTAC.

Fig. 5. Chemical structures of CRABP-targeting PROTACs.

Despite great progress being made in the development of peptide-based PROTACs that recruit VHL E3 ubiquitin ligase, the road towards small-molecule PROTACs that recruit the same complex was uncertain. However, in 2012, Crews and Ciulli found the first small-molecule ligands for VHL that possessed better physicochemical properties compared to the peptide-based ones as well as adequate affinities for this E3 ubiquitin ligase.[61]

To date, none of the reported PROTACs had been characterized *in vivo*. In 2013, the PhosphoPROTAC molecules emerged. They provided the first evidence that PROTACs could perform biological effects *in vivo* since phosphatidylinositol 3-kinase (PI3K)-targeting PhosphoPROTAC designated as $^{\mathrm{ErbB2}}\mathrm{PP}_{\mathrm{PI3K}}$ (**12**, Fig. 6) inhibited tumor growth in murine models.[62] At the *C*-terminus, the mentioned PhosphoPROTAC was composed of the peptide sequence (marked in blue) derived from the transcription factor HIF-1*α*, which enables binding to the VHL E3 ubiquitin ligase. The sequence downwards (marked in brown) is the octa-*D*-arginine motif that enabled cell permeability of the peptide *via* the same mechanism used by the structurally related HIV transactivator of transcription

(CC) 2022 SCS.

(Tat) protein. The *N*-terminus contains a 24-amino acid sequence (marked in red) taken from a PI3K-binding domain on the intracellular region of ErbB3. After phosphorylation of dual tyrosine residues (underlined) in this motif by ErbB2, the 24-amino acid sequence binds to PI3K and ultimately directs it towards polyubiquitination and degradation.

H2N-GPGGDYAAMGACPASEQGYEEMRA-NH-(CH2CH2O)3-C(O)-ALAPYIP-(*D*-R)8-CONH2

12

Fig. 6. Chemical structure of PI3K-targeting PhosphoPROTAC.

The applicability of the above-mentioned small-molecule-based VHL ligand was first demonstrated in 2015 in the making of HaloPROTAC molecules.[63] Namely, these ones are composed of the small-molecule VHL ligand and a chloroalkane linker, which enables covalent bonding between HaloPROTAC and HaloTag7 (HT7). The latter was a modified bacterial dehalogenase that covalently binds to hexyl chloride moieties.[64] The synthesized HaloPROTACs successfully recruited VHL E3 ubiquitin ligase and induced the degradation of green fluorescent protein (GFP)-HT7 fusion protein. The most potent HaloPROTAC molecule and one of the most potent PROTACs of all the designated as HaloPROTAC 3 (**13**, Fig. 7) performed D_{max} (maximum percentage of target protein degraded) of 90 % and DC_{50} (concentration at which the target protein is degraded by 50 %) of 19 nM toward GFP-HT7 fusion protein.[63]

13

Fig. 7. Chemical structure of GFP-HT7-targeting HaloPROTAC.

Another example of a PROTAC based on a similar small-molecule VHL ligand, marked as VH032, is MZ1 (**14**, Fig. 8), which was discovered in 2015. It performed selective removal of BRomoDomain-containing protein (BRD) 4 over BRD2 and BRD3,[40] all of which belong to a family of BET (Bromodomain and Extra-Terminal domain) proteins. During the same year, Bradner and colleagues[65] synthesized dBET1 (**15**, Fig. 8) that contains a thalidomide derivative as a CRL4^{CRBN} (cereblon) E3 ubiquitin ligase recruiter. It induced the degrad-

(CC) 2022 SCS.

ation of BRD4 with a DC_{50} of 430 nM, which is important for the growth and survival of cancer cells.

14 15

Fig. 8. Chemical structures of (+)-JQ1-based BRD4-targeting PROTACs.

Hence, the discovery of small-molecule PROTACs with drug-like properties enabled the emergence of potent and cell-permeable PROTACs. The third small--molecule-based PROTAC molecule (**16**, Fig. 9) reported in 2015 was developed in the laboratory of Crews with the collaboration of GlaxoSmithKline. It induced the selective degradation of receptor-interacting serine/threonine-protein kinase 2 (RIPK2) protein with low nanomolar DC_{50} of 1.4 nM.[47]

16

Fig. 9. Chemical structure of RIPK2-targeting PROTAC.

Additionally, mechanistic characterization using an *in vitro* ubiquitination assay confirmed that this PROTAC performs a catalytic MOA. Furthermore, the analogous PROTAC, which contains a stereoisomer of the VHL ligand unable to recruit the VHL E3 ubiquitin ligase, was used as a negative control. Since the PROTAC modified in this way was unable to induce RIPK2 degradation, it was confirmed that behind this biological effect stands a mechanism dependent on the E3 ubiquitin ligase.

It is noteworthy that PROTACs are beyond the rule-of-five (bRo5) compounds,[66] meaning that the molar masses of PROTACs are in a significantly higher range than the guidelines of Lipinski. Additionally, such molecules possess high polar surface area (PSA) and all these issues are associated with poor cell permeability, poor solubility and limitation of other drug-like properties. Hence, in 2016 Heightman and colleagues[67] conceived the idea about PROTACs that could be formed intracellularly from two small precursors able to pass through cellular membranes easier than one large compound (*i.e.*, PROTAC).

(CC) 2022 SCS.

The molecules arising from this concept were called in-cell CLIck-formed proteolysis targeting chimeras (CLIPTACs). This means that these ones were formed *via* bioorthogonal "click" reaction between the corresponding smaller precursors inside living cells, which overcomes unfavourable physicochemical properties often seen with conventional PROTACs. The reported CLIPTACs successfully degraded two key oncology targets, BRD4 (**17**, Fig. 10) and extracellular signal-regulated kinases (ERK1/2, **18**, Fig. 10). The CLIPTACs were formed after the "click" reaction between the tetrazine-tagged thalidomide derivative and the corresponding *trans*-cyclooctene-tagged POI ligands: (+)-JQ1 for BRD4 and covalent ERK1/2 inhibitor for ERK1/2 (Fig. 10).

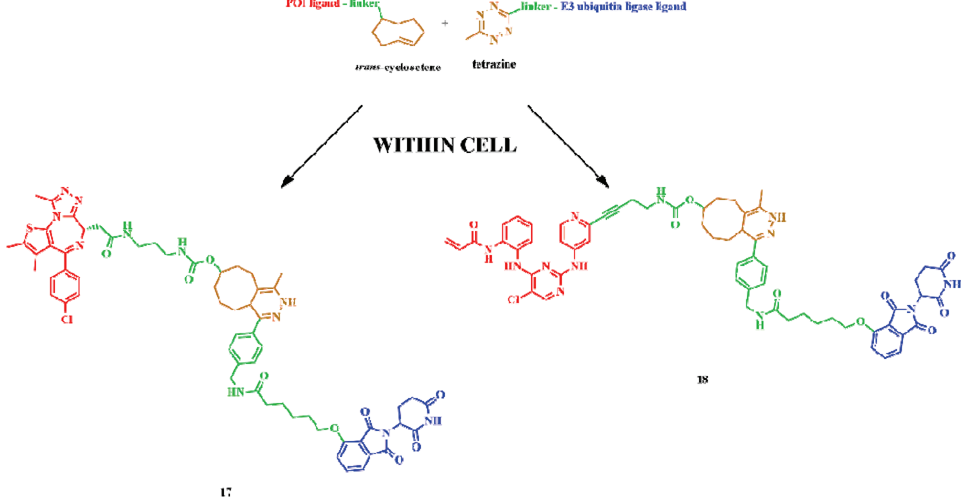

Fig. 10. Formation and chemical structures of CLIPTACs targeting BRD4 and ERK1/2.

Beside (+)-JQ1, additional BET inhibitors were used for making potent degraders. One of them, marked as HJB97, was utilized for making BETd-260 degrader (**19**, Fig. 11) reported in 2018, which was capable of inducing the degradation of BRD4 at concentrations as low as 30 pM in the RS4;11 cell line within a 24 h treatment.[68]

19

Fig. 11. Chemical structure of HJB97-based BRD4-targeting PROTAC.

(CC) 2022 SCS.

Ultimately, some PROTACs could potentially hit the clinic as some clinical trials are currently underway. For example, ARV-110 (**20**, Fig. 12), an orally bioavailable, small-molecule PROTAC entered clinical trials in March 2019 for the treatment of patients with metastatic castration resistant prostate cancer (mCRPC) and currently is in phase II.[69] This molecule potently degrades AR with a DC_{50} of 1 nM in VCaP cells.[70] Another PROTAC, ARV-471 (**21**, Fig. 12), an orally bioavailable, small-molecule PROTAC, also entered clinical trials in August 2019 to be examined alone and in combination with palbociclib in patients with ER+/HER2-locally advanced or metastatic breast cancer (mBC). This molecule, as well as ARV-110, is currently in phase II[71] and it induces ER degradation in multiple ER+ breast cancer cell lines, including MCF-7 cells and ESR1-mutant lines with a DC_{50} of 1.8 nM in MCF-7 cells.[70]

Fig. 12. Chemical structures of AR- and ER-targeting PROTACs that have entered clinical trials.

4.3. Consideration of the physicochemical properties of PROTACs

The design of PROTAC molecules is challenging since linker-mediated chemical bonding of two molecules with drug-like properties ultimately gives a hybrid molecule with physicochemical properties beyond the Lipinski rule of five.[72,73] It potentially can interfere with cell permeability and oral bioavailability.[74] Despite this, there are numerous examples of PROTACs achieving passive membrane permeability and oral bioavailability.[75] Additionally, the Arvinas company reported that in a preclinical tauopathy model, the PROTAC directed toward the tau protein crosses the blood-brain barrier.[76] Thus, strict adherence to the Lipinski rule of five is not always a good strategy in medicinal chemistry.

A paper published by Maple and colleagues[74] presents a comprehensive database of degrader structures from the peer reviewed literature. More precisely, the aim of this publication was to present physicochemical properties of numerous reported PROTACs and to connected them with biological activities. Physicochemical properties were presented through molecular descriptors and biological activities through degrader scores (*Deg_S*). Five parameters were considered for calculating *Deg_S*: DC_{50}, D_{max}, observed degradation, degrader concentration and incubation time. In order to generate the *Deg_S* values, all scores were summed and normalized against the total number of parameters included for each degrader. The degraders included in the present study could be characterized by a range of high interval molecular descriptors: molecular weight

(CC) 2022 SCS.

(*MW*) 614–1,413, calculated log *P* (clog *P*) –2.7–9, number of hydrogen bond donors (*HBD*) 1–10, number of hydrogen bond acceptors (*HBA*) 8–23, number of rotatable bonds (*NRB*) 6–49, number of aromatic rings (*NAR*) 1–7 and topological polar surface area (*TPSA*) 124–389. However, despite relatively high *MW*, the mean *HBD* was ≤ 5, which is within the Lipinski rule of five. The only conclusion that could be drawn by observing the mean vales of the molecular descriptors and *Deg_S* values was that clog *P* increases with increasing *Deg_S*. Correlations between *Deg_S* and other molecular descriptors (*i.e.*, physicochemical properties) were not found. This was expected since the used dataset incorporated a wide range of degraders containing diverse POI ligands, E3 ubiquitin ligase ligands and linkers. Furthermore, PROTACs efficacy depends on their pharmacological properties, such as the kinetics of TC formation as well as POI degradation and resynthesis rate.[77]

Maple and colleagues compared physicochemical properties of the most active PROTACs with other molecules belonging to bRo5 space. Those ones were drugs and clinical candidates with molecular weight greater than 500 Da,[78] orally available preclinical compounds breaking more than one of the Lipinski rules[79] and orally available macrocyclic drugs.[80] It was concluded that PROTACs belong to the physicochemical space which is different from the one to which all of the before mentioned classes of drugs belong. Furthermore, the mean HBD matches with the Lipinski rules and the TPSA values were lower than those for all other bRo5 molecules, which may lead to the conclusion that this molecular descriptor be monitored during PROTACs development. Since highly flexible linkers are usually incorporated into PROTAC molecules, their NRB is greater when compared to the one present within other bRo5 compounds. Noticeable flexibility of PROTACs achieved by great NRB along linkers is potentially significant for cell permeability since recent analysis suggests that dynamically exposed polarity is important for cell permeability and solubility of bRo5 compounds.[81,82]

4.4. The influence of linkers on the physicochemical and pharmacological properties of PROTACs

It is important to note that linkers are not just “ropes” connecting ligands for POI and E3 ubiquitin ligases, their roles are much more complex (see Supplementary material to this paper, Section S-4). Currently there are no general rules regarding the design of the linker the application of which would certainly lead to potent degraders containing any pair of POI/E3 ubiquitin ligase ligands – the whole process is mainly based on the trial and error method. However, when analyzing reported PROTACs some combinations of main structural motifs could be noticed. More precisely, the most common types of linkers are PEG-, alkyl- or modified glycol-based as well as linkers containing more rigid motifs, such as

Available on line at www.shd.org.rs/JSCS/

(CC) 2022 SCS.

alkyne, piperazine, piperidine and triazole. A summary of different types of linkers is outlined in Table I.[83]

TABLE I. Common structural motifs of linkers and their key features

Linker structure	Structural motif	Key features
	PEG sequence	- Synthetic feasibility and commercial availability - Linker length can be precisely tuned - - Flexibility
	Alkyl sequence	
	Modified glycol sequence	
	Alkyne	- Possible PROTACs potency increase - Possible improvement in PROTACs physicochemical properties - Restricted PROTACs conformation
	Piperazine	
	Piperidine	
	Triazole	- Facilitation in PROTACs syntheses - Possible high-yielding PROTACs syntheses - Possibility to establish additional hydrogen bonds within TC

Linkers containing PEG, alkyl or modified glycol sequences are the most common within PROTACs since they are feasible and flexible. In addition, their length and chemical composition could be modified using common synthetic methods. Combination of PEG and alkyl motifs for making linkers enable tuning of some important physicochemical properties which could be precisely described through clog *P* and TPSA. Values of those ones are in a direct connection with both solubility and cell permeability.

Linkers containing flexible structural motifs were in some PROTACs replaced with those ones containing more rigid elements, such as alkyne or various heterocyclic compounds, in order to increase the rigidity of the final molecule. Improvement in the biological activity of PROTACs by introducing groups that increase the rigidity can be illustrated on BET-targeting PROTACs.[84] Namely, the PROTAC containing an alkyl moiety connected to lenalidomide (CRBN ligand, **22**, Table II) displayed picomolar activity in three leukemia cell lines (MV4;11, MOLM13 and RS4;11). By replacing the amine with an alkyne linkage within thalidomide derivative, a very potent PROTAC denoted as QCA570 (**23**, Table II) was obtained. The resulting molecule showed 6- and 3-fold increased cell activity toward MV4;11 and MOLM13 cells.

(CC) 2022 SCS.

TABLE II. Comparative presentation of IC_{50} for two PROTACs containing a flexible and a rigidified linker, respectively

22	23
MV4;11: 51 pM (MV4;11) MOLM13: 180 pM (MOLM13) RS4;11: 1.2 pM (RS4;11)	MV4;11: 8.3 pM MOLM13: 62 pM RS4;11: 32 pM

Using the rigidification strategy, a potent AR degrader denoted as ARD-69 (**24**, Table III) was discovered.[85] Namely, introducing of an ionisable linker containing two adjacent piperidines in the vicinity of an alkyne makes the resultant PROTAC more water soluble compared to the analogous PROTACs containing hydrocarbon-based linkers.

TABLE III. The chemical structure of ARD-69 and its degradation activity represented through DC_{50}

24
LNCaP: 0.86 nM VCaP: 0.76 nM

The before-mentioned PROTAC containing a very rigid linker displayed subnanomolar biological activity at degrading AR in LNCaP and VCaP prostate cancer cell lines. Furthermore, downregulation of AR-mediated transcription in the same cell lines was achieved. This highlights the advantages of rigid and polar linkers containing alkynes and heterocyclic motifs with regard to those containing PEG, alkyl or modified glycol sequences. The advantages of the former ones could be explained through their ability to improve the pharmacokinetic properties as well as to rigidify the conformation of PROTACs, which lead to the formation of functional TCs.

(CC) 2022 SCS.

Triazole-containing linkers are common in reported PROTACs.[48] The reason for this could be found both in the straightforward triazole synthesis using "click" reaction as well as in chemical robustness of this heterocycle to metabolism.[86] The copper-catalysed Huisgen 1,3-dipolar cycloaddition, often referred as "click" reaction, between an alkyne and an azide has been widely used in the synthesis of triazole derivatives. This is generally a high yielding reaction that shows great regioselectivity toward 1,4-disubstituted 1,2,3-triazoles.[87] Hence, this reaction could be employed for the straightforward synthesis of PROTACs using an alkyne functionality connected to one ligand and an azide functionality connected to the other. Triazole-containing linkers, in addition to be used for feasible PROTACs synthesis, could also be used to modify their physicochemical properties as well as for establishing additional interactions within TC that could stabilize it. More precisely, Schiedel and colleagues[88] developed a triazole-containing SirReal (Sirtuin Rearranging ligand) (**25**, Table IV) that showed improved aqueous solubility compared to the parent compound (**26**, Table IV). Furthermore, the cocrystal structure of Sirtuin 2 (Sirt2) complexed with **25** (PDB code: 5DY5) unveiled that triazole moiety protrudes into the binding channel for acetyllysine where it forms a hydrogen bond with R97 of Sirt2. This resulted in a more efficient blockage of the substrate binding site.

TABLE IV. SirReals' IC_{50} data regarding Sirt2

25	**26**
0.16 μM	3.75 μM

More important, based on the previous study of SirReal-based affinity probe,[89] Schiedel and colleagues[88] had reliable information that placing a linker at N1 of the triazole of SirReal does not diminish the affinity of the resulting PROTAC toward Sirt2. Therefore, the alkyne-functionalized SirReal **27** was "clicked" with the azide-functionalized thalidomide derivative **28** to obtain the corresponding PROTAC **29** (Fig. 13).

It is important to emphasize that docking analysis of **29** in the TC with Sirt2 and CRBN confirmed that the before-mentioned hydrogen bonding originating from the triazole ring remains conserved. More important, the resulting PROTAC **29** inhibited Sirt2 almost 10-fold more potent than the starting alkyne **27**, which

(CC) 2022 SCS.

confirms the contribution of the hydrogen bond originating from the triazole of PROTAC to the overall inhibitory potential.

Fig. 13. Synthesis of a Sirt2-targeting PROTAC using a "click" reaction.

4.5. Advantages of the PROTAC -induced POI degradation over the current therapeutic modalities

In the past few decades, several advanced pharmacological approaches have emerged in order to target diseases more efficiently. The traditional inhibitor-based paradigm was modernized by the emergence of methods to block extracellular signaling using mAbs as well as by degrading target mRNA using RNAi approaches.[5]

The crucial advantage of mAbs-based therapies comes from their high binding affinity toward corresponding biological targets as well as a prolonged pharmacokinetic profile due to their endosomal recycling. The main therapeutic application of mAbs isthe blocking of extracellular protein-protein or protein-ligand interactions. However, the main disadvantages of mAbs are their inability to cross cell membanes, the need for parenteral administration and expensive production.[5]

As opposed to mAbs, RNAi molecules (*e.g.*, siRNAs) can pass through cell membranes. Additionally, these molecules can have great pharmacological effects toward mRNAs. Due to their catalytic MOA, siRNAs perform prolonged degradation of mRNAs and display efficacy even at low concentrations, because

(CC) 2022 SCS.

each siRNA can induce the degradation of multiple mRNA transcripts. However, RNAi molecules have certain shortcomings, such as poor oral bioavailability, poor pharmacokinetics and limited tissue distribution. However, both mAbs and RNAi molecules can affect therapeutically relevant targets more efficiently than ligands belonging to the ODP concept.[5]

Genetic engineering techniques, such as CRISPR-Cas9, usually have a long cycle, irreversible MOA and high cost of goods. All this makes them unsuitable for use in research. Unlike such genetic engineering techniques, PROTACs degrade POIs directly without acting on the genome level. Furthermore, PROTACs provide elegant temporal control over POI degradation. More precisely, such molecules have biological effects over a specific time frame, which enables the recovery of degraded POI (*i.e.*, resynthesis) after discontinuation of the treatment. Thus, as a new method for expeditious POI degradation that *via* influencing the PROTAC concentration can be temporally controlled, PROTAC technology could be perceived as a strategy for reversible and controlled POI degradation, which is an additional and complementary technique to the existing genetic engineering techniques.[90]

On the basis of all the above, it can be concluded that combining the features of traditional inhibitors, mAbs, siRNAs and CRISPR-Cas9 would provide almost ideal molecules. Briefly, such molecules would have the ability to target intracellular proteins and proteins considered pharmacologically intractable, possess high selectivity, oral bioavailability as well as adequate distribution into diverse tissues (including CNS) and perform catalytic MOA which would enable adequate biological effects at low concentrations.[22] The novel pharmacological approach that possess many advantages over the other ones and that could address all previously mentioned features is PROTAC-mediated IPD[91] (see Supplementary material, Section S-5). The summary of the before-mentioned pharmacological modalities is given in Table V.[5,92,93]

TABLE V. The properties of five different pharmacological modalities; PO – oral; IV – intravenous; SC – subcutaneous; IM – intramuscular; IP – intraperitoneal

Property	Traditional inhibitors	mAbs	siRNAs	CRISPR-Cas9	IPD
Effect on intracellular targets	Yes	No	Yes	Yes	Yes
Systemic delivery	Yes	Yes	No	Yes	Yes
Tissue penetration	Yes	Poor	Poor	Yes	Yes
Effect on scaffold proteins	No	Yes	Yes	Yes	Yes
Elimination of pathogenic proteins	No	No	Yes	Yes	Yes
Oral bioavailability	Yes	No	No	No	Yes
The ability to achieve high potency and selectivity	Poor	Yes	Yes	Yes	Yes
Catalytic MOA	No	No	Yes	Yes	Yes
Route of administration	PO/IV/SC	IV/SC	IV/SC	IV/IM/IP	PO/IV/SC

Available on line at www.shd.org.rs/JSCS/

(CC) 2022 SCS.

5. CONCLUSIONS AND PERSPECTIVES

Many questions could be asked by observing the PROTAC molecule:[1] a) whether the molecule will enter the cell?; b) whether it will be metabolically stable?; c) whether a rational structure–activity relationship (SAR) study could be performed?; d) whether the molecule will be soluble in water?; e) will it be selective in protein degradation?; f) will it be safe?; g) will it follow the Lipinski rule of five?; h) will chemical synthesis be feasible and cost-effective?; i) can PROTAC technology be applied on a wide range of POIs?

However, the PROTAC technology could have its greatest impact by developing molecules that act on the proteome currently considered as pharmacologically intractable. Examples of PROTAC-mediated degradations so far have been applied to diverse POIs, such as proteases, nuclear hormone receptors, epigenetic factors and kinases. Thus, the PROTAC technology has the potential to target about 80 % of the proteome.[94] However, PROTACs suffer from many shortcomings, such as long synthetic sequences, possible presence of multiple stereocenters, demanding physicochemical characterization and potential for non-crystallinity. Hence, each of those drawbacks has the potential to obstruct the development of PROTAC. Despite this, all the stated results confirm that molecules originating from the EDP concept could have great impact on further drug development and that PROTAC technology could become an important tool in medicinal chemistry thereby making the pharmacologically undruggable proteome pharmacologically vulnerable.[11]

Acknowledgments. This research was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia through the Grant Agreement with the University of Belgrade – Faculty of Pharmacy No. 451-03-68/2022-14/200161 and by the Science Fund of the Republic of Serbia through the Serbian Science and Diaspora Collaboration Program: Knowledge Exchange Vouchers (Grant No.: 6463913, acronym: DeSyHPRO).

И З В О Д

ДЕГРАДАЦИЈА ПРОТЕИНА ИНДУКОВАНА PROTAC МОЛЕКУЛИМА КАО НОВА СТРАТЕГИЈА У РАЗВОЈУ ЛЕКОВА

МЛАДЕН КОРАВОВИЋ[1], БОЈАН МАРКОВИЋ[2], МИЛЕНА КОВАЧЕВИЋ[3], МИЛЕНА РМАНДИЋ[4] и ГОРДАНА ТАСИЋ[1]

[1]*Универзитет у Београду – Фармацеутски факултет, Катедра за органску хемију, Војводе Степе 450, 11221 Београд,* [2]*Универзитет у Београду – Фармацеутски факултет, Катедра за фармацеутску хемију, Војводе Степе 450, 11221 Београд,* [3]*Универзитет у Београду – Фармацеутски факултет, Катедра за фармакокинетику и клиничку фармацију, Војводе Степе 450, 11221 Београд и* [4]*Универзитет у Београду – Фармацеутски факултет, Катедра за аналитику лекова, Војводе Степе 450, 11221 Београд*

Традиционални концепт открића лекова базиран је на фармаколошком моделу заснованом на окупираности циљних протеина. То подразумева развој инхибитора који окупирају везујућа места директно повезана са функцијама протеина. Стога, протеини који немају таква везујућа места се генерално сматрају фармаколошки недодирљивим. Осим тога, лекови који делују на овакав начин морају се примењивати у режимима

(CC) 2022 SCS.

дозирања који често доводе до претеране системске изложености леку ради одржања довољне инхибиције протеина. Дакле, постоји ризик од појаве везивања лека ван свог примарног места дејства и нежељених ефеката. Окосница развоја лекова је значајно измењена откако су се појавили PROTAC (енг. *proteolysis targeting chimera*) молекули пре двадесет година као део фармаколошког модела заснованог на покретању догађаја који доводе до разградње циљних протеина. Ово су бифункционални молекули који користе убиквитин-протеазом систем, а састоје се од лиганда који се везује за протеин од интереса (POI), лиганда који регрутује E3 убиквитин лигазу (E3UL) и линкера који повезује ова два дела. Фармаколошки, PROTAC молекули доводе POI и E3UL у близину, што води формирању функционалног тернарног комплекса POI–PROTAC–E3UL. Овај догађај води полиубиквитинацији и следственој деградацији POI 26S протеазомом. Развој и изузетна својства PROTAC молекула која су их довела до клиничких студија дискутовани су овом раду.

(Примљено 9. децембра 2021, ревидирано 9. марта, прихваћено 14. марта 2022)

REFERENCES

1. I. Churcher, *J. Med. Chem.* **61** (2018) 444 (http://dx.doi.org/10.1021/acs.jmedchem.7b01272)
2. L. E. Limbird, *Mol. Interv.* **4** (2004) 326 (http://dx.doi.org/10.1124/mi.4.6.6)
3. M. Benchekroun, *Future Drug. Discov.* **1** (2019) FDD16 (http://dx.doi.org/10.4155/fdd-2019-0019)
4. M. Rask-Andersen, M. S. Almén, H. B. Schiöth, *Nat. Rev. Drug Discov.* **10** (2011) 579 (http://dx.doi.org/10.1038/nrd3478)
5. T. K. Neklesa, J. D. Winkler, C. M. Crews, *Pharmacol. Ther.* **174** (2017) 138 (http://dx.doi.org/10.1016/j.pharmthera.2017.02.027)
6. J. Campbell, C. J. Ryan, R. Brough, I. Bajrami, H. N. Pemberton, I. Y. Chong, S. Costa-Cabral, J. Frankum, A. Gulati, H. Holme, R. Miller, S. Postel-Vinay, R. Rafiq, W. Wei, C. T. Williamson, D. A. Quigley, J. Tym, B. Al-Lazikani, T. Fenton, R. Natrajan, S. J. Strauss, A. Ashworth, C. J. Lord, *Cell Rep.* **14** (2016) 2490 (http://dx.doi.org/10.1016/j.celrep.2016.02.023)
7. G. S. Cowley, B. A. Weir, F. Vazquez, P. Tamayo, J. A. Scott, S. Rusin, A. East-Seletsky, L. D. Ali, W. F. Gerath, S. E. Pantel, P. H. Lizotte, G. Jiang, J. Hsiao, A. Tsherniak, E. Dwinell, S. Aoyama, M. Okamoto, W. Harrington, E. Gelfand, T. M. Green, M. J. Tomko, S. Gopal, T. C. Wong, H. Li, S. Howell, N. Stransky, T. Liefeld, D. Jang, J. Bistline, B. Hill Meyers, S. A. Armstrong, K. C. Anderson, K. Stegmaier, M. Reich, D. Pellman, J. S. Boehm, J. P. Mesirov, T. R. Golub, D. E. Root, W. C. Hahn, *Sci. Data* **1** (2014) 140035 (http://dx.doi.org/10.1038/sdata.2014.35)
8. T. Wang, K. Birsoy, N. W. Hughes, K. M. Krupczak, Y. Post, J. J. Wei, E. S. Lander, D. M. Sabatini, *Science* **350** (2015) 1096 (http://dx.doi.org/10.1126/science.aac7041)
9. J. Barretina, G. Caponigro, N. Stransky, K. Venkatesan, A. A. Margolin, S. Kim, C. J. Wilson, J. Lehár, G. V. Kryukov, D. Sonkin, A. Reddy, M. Liu, L. Murray, M. F. Berger, J. E. Monahan, P. Morais, J. Meltzer, A. Korejwa, J. Jané-Valbuena, F. A. Mapa, J. Thibault, E. Bric-Furlong, P. Raman, A. Shipway, I. H. Engels, J. Cheng, G. K. Yu, J. Yu, P. Aspesi, M. de Silva, K. Jagtap, M. D. Jones, L. Wang, C. Hatton, E. Palescandolo, S. Gupta, S. Mahan, C. Sougnez, R. C. Onofrio, T. Liefeld, L. MacConaill, W. Winckler, M. Reich, N. Li, J. P. Mesirov, S. B. Gabriel, G. Getz, K. Ardlie, V. Chan, V. E. Myer, B. L. Weber, J. Porter, M. Warmuth, P. Finan, J. L. Harris, M. Meyerson, T. R. Golub, M. P.

(CC) 2022 SCS.

Morrissey, W. R. Sellers, R. Schlegel, L. A. Garraway, *Nature* **483** (2012) 603 (http://dx.doi.org/10.1038/nature11003)
10. A. L. Hopkins, C. R. Groom, *Nat. Rev. Drug Discov.* **1** (2002) 727 (http://dx.doi.org/10.1038/nrd892)
11. A. C. Lai, C. M. Crews, *Nat. Rev. Drug Discov.* **16** (2017) 101 (http://dx.doi.org/10.1038/nrd.2016.211)
12. M. Toure, C. M. Crews, *Angew. Chem. Int. Ed. Engl.* **55** (2016) 1966 (http://dx.doi.org/10.1002/anie.201507978)
13. J. S. Lazo, E. R. Sharlow, *Annu. Rev. Pharmacol. Toxicol.* **56** (2016) 23 (http://dx.doi.org/10.1146/annurev-pharmtox-010715-103440)
14. L. Jin, W. Wang, G. Fang, *Annu. Rev. Pharmacol. Toxicol.* **54** (2014) 435 (http://dx.doi.org/10.1146/annurev-pharmtox-011613-140028)
15. A. A. Adjei, *J. Clin. Oncol.* **24** (2006) 4054 (http://dx.doi.org/10.1200/JCO.2006.07.4658)
16. S.-L. Paiva, C. M. Crews, *Curr. Opin. Chem. Biol.* **50** (2019) 111 (http://dx.doi.org/10.1016/j.cbpa.2019.02.022)
17. S. An, L. Fu, *EBioMedicine* **36** (2018) 553 (http://dx.doi.org/10.1016/j.ebiom.2018.09.005)
18. D. Leiser, B. Pochon, W. Blank-Liss, P. Francica, A. A. Glück, D. M. Aebersold, Y. Zimmer, M. Medová, *FEBS Lett.* **588** (2014) 653 (http://dx.doi.org/10.1016/j.febslet.2013.12.025)
19. J. Spiegel, P. M. Cromm, G. Zimmermann, T. N. Grossmann, H. Waldmann, *Nat. Chem. Biol.* **10** (2014) 613 (http://dx.doi.org/10.1038/nchembio.1560)
20. S. Dogan, R. Shen, D. C. Ang, M. L. Johnson, S. P. D'Angelo, P. K. Paik, E. B. Brzostowski, G. J. Riely, M. G. Kris, M. F. Zakowski, M. Ladanyi, *Clin. Cancer Res.* **18** (2012) 6169 (http://dx.doi.org/10.1158/1078-0432.CCR-11-3265)
21. K. Eisermann, D. Wang, Y. Jing, L. E. Pascal, Z. Wang, *Transl. Androl. Urol.* **2** (2013) 137 (http://dx.doi.org/ 10.3978/j.issn.2223-4683.2013.09.15)
22. C. M. Crews, *Chem. Biol.* **17** (2010) 551 (http://dx.doi.org/10.1016/j.chembiol.2010.05.011)
23. J. P. Overington, B. Al-Lazikani, A. L. Hopkins, *Nat. Rev. Drug Discov.* **5** (2006) 993 (http://dx.doi.org/10.1038/nrd2199)
24. J. S. Duncan, M. C. Whittle, K. Nakamura, A. N. Abell, A. A. Midland, J. S. Zawistowski, N. L. Johnson, D. A. Granger, N. V. Jordan, D. B. Darr, J. Usary, P.-F. Kuan, D. M. Smalley, B. Major, X. He, K. A. Hoadley, B. Zhou, N. E. Sharpless, C. M. Perou, W. Y. Kim, S. M. Gomez, X. Chen, J. Jin, S. V. Frye, H. S. Earp, L. M. Graves, G. L. Johnson, *Cell* **149** (2012) 307 (http://dx.doi.org/10.1016/j.cell.2012.02.053)
25. T. Visakorpi, E. Hyytinen, P. Koivisto, M. Tanner, R. Keinänen, C. Palmberg, A. Palotie, T. Tammela, J. Isola, O. P. Kallioniemi, *Nat. Genet.* **9** (1995) 401 (http://dx.doi.org/10.1038/ng0495-401)
26. G. Hatzivassiliou, K. Song, I. Yen, B. J. Brandhuber, D. J. Anderson, R. Alvarado, M. J. C. Ludlam, D. Stokoe, S. L. Gloor, G. Vigers, T. Morales, I. Aliagas, B. Liu, S. Sideris, K. P. Hoeflich, B. S. Jaiswal, S. Seshagiri, H. Koeppen, M. Belvin, L. S. Friedman, S. Malek, *Nature* **464** (2010) 431 (http://dx.doi.org/10.1038/nature08833)
27. S. J. Heidorn, C. Milagre, S. Whittaker, A. Nourry, I. Niculescu-Duvas, N. Dhomen, J. Hussain, J. S. Reis-Filho, C. J. Springer, C. Pritchard, R. Marais, *Cell* **140** (2010) 209 (http://dx.doi.org/10.1016/j.cell.2009.12.040)

(CC) 2022 SCS.

28. P. I. Poulikakos, C. Zhang, G. Bollag, K. M. Shokat, N. Rosen, *Nature* **464** (2010) 427 (http://dx.doi.org/10.1038/nature08902)
29. J. Rauch, N. Volinsky, D. Romano, W. Kolch, *Cell Commun. Signal.* **9** (2011) 23 (http://dx.doi.org/10.1186/1478-811X-9-23)
30. X. Tan, N. Thapa, Y. Sun, R. A. Anderson, *Cell* **160** (2015) 145 (http://dx.doi.org/10.1016/j.cell.2014.12.006)
31. I. Vivanco, Z. C. Chen, B. Tanos, B. Oldrini, W.-Y. Hsieh, N. Yannuzzi, C. Campos, I. K. Mellinghoff, *Elife* **3** (2014) e03751 (http://dx.doi.org/10.7554/eLife.03751)
32. Z. Weihua, R. Tsan, W.-C. Huang, Q. Wu, C.-H. Chiu, I. J. Fidler, M.-C. Hung, *Cancer Cell* **13** (2008) 385 (http://dx.doi.org/10.1016/j.ccr.2008.03.015)
33. P. M. Cromm, C. M. Crews, *Cell Chem. Biol.* **24** (2017) 1181 (http://dx.doi.org/10.1016/j.chembiol.2017.05.024)
34. A. M. Giannetti, in *Methods Enzymol.*, L. C. Kuo (Ed.), Academic Press, Cambridge, MA, 2011, p. 169 (http://dx.doi.org/10.1016/B978-0-12-381274-2.00008-X)
35. S. B. Shuker, P. J. Hajduk, R. P. Meadows, S. W. Fesik, *Science* **274** (1996) 1531 (http://dx.doi.org/10.1126/science.274.5292.1531)
36. E. H. Mashalidis, P. Śledź, S. Lang, C. Abell, *Nat. Protoc.* **8** (2013) 2309 (http://dx.doi.org/10.1038/nprot.2013.130)
37. D. A. Erlanson, S. W. Fesik, R. E. Hubbard, W. Jahnke, H. Jhoti, *Nat. Rev. Drug Discov.* **15** (2016) 605 (http://dx.doi.org/10.1038/nrd.2016.109)
38. J.-P. Renaud, C. Chung, U. H. Danielson, U. Egner, M. Hennig, R. E. Hubbard, H. Nar, *Nat. Rev. Drug Discov.* **15** (2016) 679 (http://dx.doi.org/10.1038/nrd.2016.123)
39. K. H. Bleicher, H.-J. Böhm, K. Müller, A. I. Alanine, *Nat. Rev. Drug Discov.* **2** (2003) 369 (http://dx.doi.org/10.1038/nrd1086)
40. R. Macarron, M. N. Banks, D. Bojanic, D. J. Burns, D. A. Cirovic, T. Garyantes, D. V. S. Green, R. P. Hertzberg, W. P. Janzen, J. W. Paslay, U. Schopfer, G. S. Sittampalam, *Nat. Rev. Drug Discov.* **10** (2011) 188 (http://dx.doi.org/10.1038/nrd3368)
41. I. Kola, J. Landis, *Nat. Rev. Drug Discov.* **3** (2004) 711 (http://dx.doi.org/10.1038/nrd1470)
42. M. Zengerle, K.-H. Chan, A. Ciulli, *ACS Chem. Biol.* **10** (2015) 1770 (http://dx.doi.org/10.1021/acschembio.5b00216)
43. M. Pettersson, C. M. Crews, *Drug Discov. Today Technol.* **31** (2019) 15 (http://dx.doi.org/10.1016/j.ddtec.2019.01.002)
44. T. Ishida, A. Ciulli, *SLAS Discov.* **26** (2021) 484 (http://dx.doi.org/10.1177/2472555220965528)
45. M. J. Bond, C. M. Crews, *RSC Chem. Biol.* **2** (2021) 725 (http://dx.doi.org/10.1039/D1CB00011J)
46. Z. S. Hann, C. Ji, S. K. Olsen, X. Lu, M. C. Lux, D. S. Tan, C. D. Lima, *Proc. Natl. Acad. Sci. U.S.A.* **116** (2019) 15475 (http://dx.doi.org/10.1073/pnas.1905488116)
47. D. P. Bondeson, A. Mares, I. E. D. Smith, E. Ko, S. Campos, A. H. Miah, K. E. Mulholland, N. Routly, D. L. Buckley, J. L. Gustafson, N. Zinn, P. Grandi, S. Shimamura, G. Bergamini, M. Faelth-Savitski, M. Bantscheff, C. Cox, D. A. Gordon, R. R. Willard, J. J. Flanagan, L. N. Casillas, B. J. Votta, W. den Besten, K. Famm, L. Kruidenier, P. S. Carter, J. D. Harling, I. Churcher, C. M. Crews, *Nat. Chem. Biol.* **11** (2015) 611 (http://dx.doi.org/10.1038/nchembio.1858)
48. L.-W. Xia, M.-Y. Ba, W. Liu, W. Cheng, C.-P. Hu, Q. Zhao, Y.-F. Yao, M.-R. Sun, Y.-T. Duan, *Future Med. Chem.* **11** (2019) 2919 (http://dx.doi.org/10.4155/fmc-2019-0159)

(CC) 2022 SCS.

49. K. M. Sakamoto, K. B. Kim, A. Kumagai, F. Mercurio, C. M. Crews, R. J. Deshaies, *Proc. Natl. Acad. Sci. U.S.A.* **98** (2001) 8554 (http://dx.doi.org/10.1073/pnas.141230798)
50. K. M. Sakamoto, K. B. Kim, R. Verma, A. Ransick, B. Stein, C. M. Crews, R. J. Deshaies, *Mol. Cell. Proteomics* **2** (2003) 1350 (http://dx.doi.org/10.1074/mcp.T300009-MCP200)
51. D. Zhang, S.-H. Baek, A. Ho, K. Kim, *Bioorg. Med. Chem. Lett.* **14** (2004) 645 (http://dx.doi.org/10.1016/j.bmcl.2003.11.042)
52. J. S. Schneekloth, F. N. Fonseca, M. Koldobskiy, A. Mandal, R. Deshaies, K. Sakamoto, C. M. Crews, *J. Am. Chem. Soc.* **126** (2004) 3748 (http://dx.doi.org/10.1021/ja039025z)
53. H. Lee, D. Puppala, E.-Y. Choi, H. Swanson, K.-B. Kim, *Chembiochem* **8** (2007) 2058 (http://dx.doi.org/10.1002/cbic.200700438)
54. A. Rodriguez-Gonzalez, K. Cyrus, M. Salcius, K. Kim, C. M. Crews, R. J. Deshaies, K. M. Sakamoto, *Oncogene* **27** (2008) 7201 (http://dx.doi.org/10.1038/onc.2008.320)
55. D. L. Buckley, C. M. Crews, *Angew. Chem. Int. Ed. Engl.* **53** (2014) 2312 (http://dx.doi.org/10.1002/anie.201307761)
56. A. R. Schneekloth, M. Pucheault, H. S. Tae, C. M. Crews, *Bioorg. Med. Chem. Lett.* **18** (2008) 5904 (http://dx.doi.org/10.1016/j.bmcl.2008.07.114)
57. L. T. Vassilev, B. T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, E. A. Liu, *Science* **303** (2004) 844 (http://dx.doi.org/10.1126/science.1092472)
58. K. Sekine, K. Takubo, R. Kikuchi, M. Nishimoto, M. Kitagawa, F. Abe, K. Nishikawa, T. Tsuruo, M. Naito, *J. Biol. Chem.* **283** (2008) 8961 (http://dx.doi.org/10.1074/jbc.M709525200)
59. Y. Itoh, M. Ishikawa, M. Naito, Y. Hashimoto, *J. Am. Chem. Soc.* **132** (2010) 5820 (http://dx.doi.org/10.1021/ja100691p)
60. K. Okuhira, N. Ohoka, K. Sai, T. Nishimaki-Mogami, Y. Itoh, M. Ishikawa, Y. Hashimoto, M. Naito, *FEBS Lett.* **585** (2011) 1147 (http://dx.doi.org/10.1016/j.febslet.2011.03.019)
61. D. L. Buckley, I. Van Molle, P. C. Gareiss, H. S. Tae, J. Michel, D. J. Noblin, W. L. Jorgensen, A. Ciulli, C. M. Crews, *J. Am. Chem. Soc.* **134** (2012) 4465 (http://dx.doi.org/10.1021/ja209924v)
62. J. Hines, J. D. Gough, T. W. Corson, C. M. Crews, *Proc. Natl. Acad. Sci. U.S.A.* **110** (2013) 8942 (http://dx.doi.org/10.1073/pnas.1217206110)
63. D. L. Buckley, K. Raina, N. Darricarrere, J. Hines, J. L. Gustafson, I. E. Smith, A. H. Miah, J. D. Harling, C. M. Crews, *ACS Chem. Biol.* **10** (2015) 1831 (http://dx.doi.org/10.1021/acschembio.5b00442)
64. R. F. Ohana, L. P. Encell, K. Zhao, D. Simpson, M. R. Slater, M. Urh, K. V. Wood, *Protein Expr. Purif.* **68** (2009) 110 (http://dx.doi.org/10.1016/j.pep.2009.05.010)
65. G. E. Winter, D. L. Buckley, J. Paulk, J. M. Roberts, A. Souza, S. Dhe-Paganon, J. E. Bradner, *Science* **348** (2015) 1376 (http://dx.doi.org/10.1126/science.aab1433)
66. G. Ermondi, D. Garcia Jimenez, M. Rossi Sebastiano, G. Caron, *ACS Med. Chem. Lett.* **12** (2021) 1056 (http://dx.doi.org/10.1021/acsmedchemlett.1c00298)
67. H. Lebraud, D. J. Wright, C. N. Johnson, T. D. Heightman, *ACS Cent. Sci.* **2** (2016) 927 (http://dx.doi.org/10.1021/acscentsci.6b00280)
68. B. Zhou, J. Hu, F. Xu, Z. Chen, L. Bai, E. Fernandez-Salas, M. Lin, L. Liu, C.-Y. Yang, Y. Zhao, D. McEachern, S. Przybranowski, B. Wen, D. Sun, S. Wang, *J. Med. Chem.* **61** (2018) 462 (http://dx.doi.org/10.1021/acs.jmedchem.6b01816)

(CC) 2022 SCS.

69. ClinicalTrials.gov, http://clinicaltrials.gov/ct2/show/NCT03888612 (accessed Nov 18, 2021)
70. ARVINAS, http://ir.arvinas.com/static-files/1667ba5f-46b6-4c6c-a868-bc1a306fdaad (accessed Nov 22, 2021)
71. Clinicaltrials.gov, http://clinicaltrials.gov/ct2/show/NCT04072952 (accessed Nov 18, 2021)
72. C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney, *Adv. Drug Deliv. Rev.* **46** (2001) 3 (http://dx.doi.org/ 10.1016/s0169-409x(00)00129-0)
73. C. A. Lipinski, *Drug Discov. Today Technol.* **1** (2004) 337 (http://dx.doi.org/10.1016/j.ddtec.2004.11.007)
74. H. J. Maple, N. Clayden, A. Baron, C. Stacey, R. Felix, *Medchemcomm* **10** (2019) 1755 (http://dx.doi.org/10.1039/C9MD00272C)
75. T. Neklesa, L. B. Snyder, R. R. Willard, N. Vitale, J. Pizzano, D. A. Gordon, M. Bookbinder, J. Macaluso, H. Dong, C. Ferraro, G. Wang, J. Wang, C. M. Crews, J. Houston, A. P. Crew, I. Taylor, *J. Clin. Oncol.* **37** (2019) 259 (http://dx.doi.org/10.1200/JCO.2019.37.7_suppl.259)
76. ARVINAS, http://ir.arvinas.com/node/7136/pdf (accessed Nov 22, 2021)
77. Y. Zhang, C. Loh, J. Chen, N. Mainolfi, *Drug Discov. Today Technol.* **31** (2019) 53 (http://dx.doi.org/10.1016/j.ddtec.2019.01.001)
78. B. C. Doak, B. Over, F. Giordanetto, J. Kihlberg, *Chem. Biol.* **21** (2014) 1115 (http://dx.doi.org/10.1016/j.chembiol.2014.08.013)
79. D. A. DeGoey, H.-J. Chen, P. B. Cox, M. D. Wendt, *J. Med. Chem.* **61** (2018) 2636 (http://dx.doi.org/10.1021/acs.jmedchem.7b00717)
80. E. A. Villar, D. Beglov, S. Chennamadhavuni, J. A. Porco, D. Kozakov, S. Vajda, A. Whitty, *Nat. Chem. Biol.* **10** (2014) 723 (http://dx.doi.org/10.1038/nchembio.1584)
81. M. Rossi Sebastiano, B. C. Doak, M. Backlund, V. Poongavanam, B. Over, G. Ermondi, G. Caron, P. Matsson, J. Kihlberg, *J. Med. Chem.* **61** (2018) 4189 (http://dx.doi.org/10.1021/acs.jmedchem.8b00347)
82. B. Kuhn, P. Mohr, M. Stahl, *J. Med. Chem.* **53** (2010) 2601 (http://dx.doi.org/10.1021/jm100087s)
83. R. I. Troup, C. Fallan, M. G. J. Baud, *Explor. Target. Antitumor Ther.* **1** (2020) 273 (http://dx.doi.org/10.37349/etat.2020.00018)
84. C. Qin, Y. Hu, B. Zhou, E. Fernandez-Salas, C.-Y. Yang, L. Liu, D. McEachern, S. Przybranowski, M. Wang, J. Stuckey, J. Meagher, L. Bai, Z. Chen, M. Lin, J. Yang, D. N. Ziazadeh, F. Xu, J. Hu, W. Xiang, L. Huang, S. Li, B. Wen, D. Sun, S. Wang, *J. Med. Chem.* **61** (2018) 6685 (http://dx.doi.org/10.1021/acs.jmedchem.8b00506)
85. X. Han, C. Wang, C. Qin, W. Xiang, E. Fernandez-Salas, C.-Y. Yang, M. Wang, L. Zhao, T. Xu, K. Chinnaswamy, J. Delproposto, J. Stuckey, S. Wang, *J. Med. Chem.* **62** (2019) 941 (http://dx.doi.org/10.1021/acs.jmedchem.8b01631)
86. H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed. Engl.* **40** (2001) 2004 (http://dx.doi.org/10.1002/1521-3773(20010601)40:11<2004::AID-ANIE2004>3.0.CO;2-5)
87. J. E. Moses, A. D. Moorhouse, *Chem. Soc. Rev.* **36** (2007) 1249 (http://dx.doi.org/10.1039/B613014N)
88. M. Schiedel, D. Herp, S. Hammelmann, S. Swyter, A. Lehotzky, D. Robaa, J. Oláh, J. Ovádi, W. Sippl, M. Jung, *J. Med. Chem.* **61** (2018) 482 (http://dx.doi.org/10.1021/acs.jmedchem.6b01872)

(CC) 2022 SCS.

89. M. Schiedel, T. Rumpf, B. Karaman, A. Lehotzky, S. Gerhardt, J. Ovádi, W. Sippl, O. Einsle, M. Jung, *Angew. Chem. Int. Ed. Engl.* **55** (2016) 2252 (http://dx.doi.org/10.1002/anie.201509843)
90. X. Sun, J. Wang, X. Yao, W. Zheng, Y. Mao, T. Lan, L. Wang, Y. Sun, X. Zhang, Q. Zhao, J. Zhao, R.-P. Xiao, X. Zhang, G. Ji, Y. Rao, *Cell Discov.* **5** (2019) 10 (http://dx.doi.org/10.1038/s41421-018-0079-1)
91. R. J. Deshaies, *Nat. Chem. Biol.* **11** (2015) 634 (http://dx.doi.org/10.1038/nchembio.1887)
92. X. Sun, H. Gao, Y. Yang, M. He, Y. Wu, Y. Song, Y. Tong, Y. Rao, *Signal Transduct. Target. Ther.* **4** (2019) 64 (http://dx.doi.org/10.1038/s41392-019-0101-6)
93. C. E. Nelson, C. H. Hakim, D. G. Ousterout, P. I. Thakore, E. A. Moreb, R. M. C. Rivera, S. Madhavan, X. Pan, F. A. Ran, W. X. Yan, A. Asokan, F. Zhang, D. Duan, C. A. Gersbach, *Science* **351** (2016) 403 (http://dx.doi.org/10.1126/science.aad5143)
94. G. A. Collins, A. L. Goldberg, *Cell* **169** (2017) 792 (http://dx.doi.org/10.1016/j.cell.2017.04.023).

(CC) 2022 SCS.