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SUPPLEMENTARY MATERIAL TO

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

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S-1. METHODS TO MANIPULATE PROTEINS OF INTEREST

One method for modulating intracellular proteins is through agents based on nucleic acids, such as antisense oligonucleotides (ASOs), as well as by using agents that exploit RNA interference (RNAi) such as small interfering RNAs (siRNAs). Agents based on nucleic acids have proved themselves to be useful in research, but their development toward potential medicines has faced many challenges: unmodified nucleotides are unstable in serum,[1] while modified ones tend to accumulate in the kidneys[2,3] and can be immunogenic,[4,5] Furthermore, agents based on nucleic acids encapsulated by nanoparticles to improve the distribution are sequestrated in the liver.[6–9] On the other hand, RNAi can recruit off-target messenger ribonucleic acid (mRNA), which leads to undesired effects.[10–12]

Innovative CRISPR-Cas9 genetic engineering technology performs direct genome modification to achieve gene knockout,[13,14] *i.e.* to make the targeted gene inoperative. Although CRISPR-Cas9 is very useful in research, its path toward clinical application is questionable.[15]

The efficacy of these approaches is strongly dependent on the target protein half-life – the long-lived ones are less affected.

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S-2. UBIQUITIN-PROTEASOME SYSTEM (UPS)

The UPS is the primary pathway for intracellular protein degradation in eukaryotic cells[16] (Fig. S-1). In this process, ubiquitin, evolutionary conserved 76 amino acid polypeptide, is used to mark proteins for degradation. After labeling with ubiquitin, proteins are recognized and degraded by the 26S proteasome. Mechanistically, protein ubiquitination occurs through a three-step enzymatic reaction.[17]

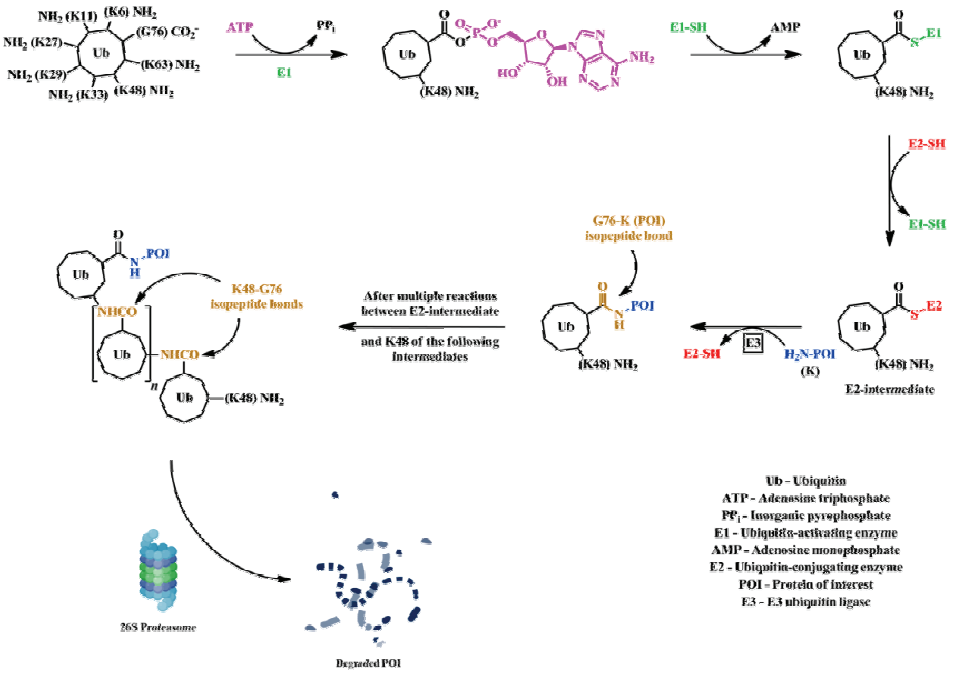

Fig. S-1. Ubiquitin-proteasome system.[18]
(K-residues on all ubiquitins except on the first one were omitted for clarity)

First, the ubiquitin-activating enzyme (E1) creates a thioester bond *via* an ATP-dependent mechanism between the *C*-terminal glycine-76 (G76) residue of ubiquitin and a cysteine residue in its active site. The thioesterified ubiquitin is then transferred from the E1 active site to the ubiquitin-conjugating enzyme (E2) *via* a transthioesterification reaction. Finally, the E3 ubiquitin ligase (E3) binds to both E2-bound ubiquitin and the protein intended for degradation promoting the transfer of ubiquitin onto the protein. After this last step, an isopeptide bond is formed between the carboxyl group of the last amino acid of ubiquitin– G76 and the ε-amino group of the substrate protein's lysine (K). Ubiquitin itself contains seven lysine residues at positions 6, 11, 27, 29, 33, 48 and 63 all of which can serve as acceptors for the second ubiquitin molecule, thus leading to the polyubi-

quitination of the substrate protein after multiple runs of ubiquitination.[19–21] Interestingly, organization of the polyubiquitination enzymatic cascade follows a hierarchical order. Namely, the human genome encodes two E1 enzymes, 38 E2 enzymes and more than 600 E3 enzymes.[21–25] It is important to say that some proteins can be ubiquitinated with only one ubiquitin on a single lysine residue, which is known as monoubiquitination, as well as on multiple lysine residues, which is known as multi-monoubiquitination. However, only polyubiquitination of proteins can lead to their degradation. Depending on the mode by which ubiquitins are attached in the polyubiquitin chain, the outcome for polyubiquitinated proteins can be different. Proteins marked with K48- or K11-linked polyubiquitin chains are predominantly targeted for recognition and degradation by the 26S proteasome, whereas the K63-linked polyubiquitination or monoubiquitination alters the function of the protein and their subcellular localization. Thus, proteins marked in this way are not intended for degradation but to participate in protein-protein interactions (PPIs), DNA damage responses and signal transduction.[26–31]

S-3. DYNAMICS OF PROTAC-MEDIATED INDUCED PROTEIN DEGRADATION (IPD)

The mechanism of IPD depends on the formation of a ternary complex (TC) that enables the protein of interest (POI) polyubiquitination and proteasomal degradation. Some mathematical models describing TC formation have already been established[32,33] and they predict a bell-shaped dependency between the concentrations of TC and PROTAC (Fig. S-2).[34]

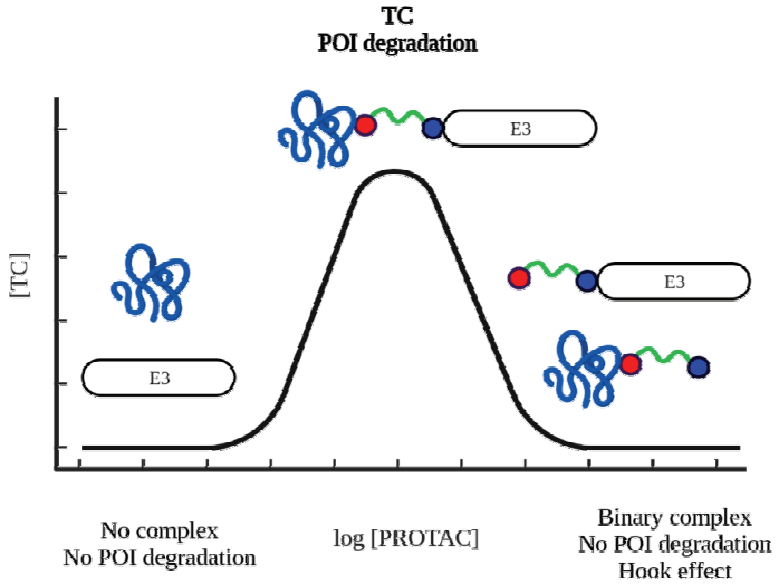

Fig. S-2. Kinetics of ternary complex formation.[35]

Therefore, when the concentration of PROTAC molecules is significantly higher than the DC_{50} (concentration at which the target protein is degraded by 50 %), autoinhibition of TC formation occurs due to increased concentration of PROTAC:E3 ubiquitin ligase and PROTAC:POI binary non-functional complexes.[36] This phenomenon is called "the hook effect"[37] and in the case of PROTACs can be defined as the reduced degradation of POI at high concentrations of small-molecule PROTACs. This phenomenon commonly occurs with three-component systems.[36]

Moreover, attractive or repulsive interactions between the POI and E3 ubiquitin ligase may affect TC formation (Fig. S-3). The term "cooperativity" (α) is used to describe these interactions. More precisely, positive cooperativity (*i.e.* $\alpha > 1$) occurs when PPIs between POI and E3 ubiquitin ligase leads to TC formation. Conversely, negative cooperativity (*i.e.* $\alpha < 1$) occurs when the before-mentioned PPIs revoke TC formation. Positive cooperativity minimizes the extent of the hook effect, thus resulting in increased productive TC formation.[38]

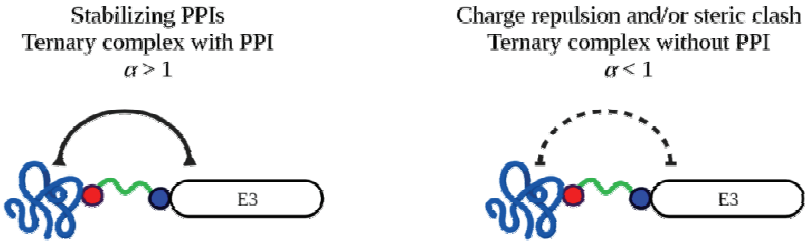

Fig. S-3. Positive and negative cooperativity.[35]

The first crystal structure of TC composed of the MZ1 PROTAC molecule **14**, BRomoDomain-containing protein (BRD) 4 protein and von Hippel-Lindau tumor suppressor (VHL) E3 ubiquitin ligase was reported in 2017 by Ciulli.[39] The crystal structure disclosed the interactions between BRD4 and VHL E3 ubiquitin ligase as well as interactions between the linker and BRD4. The presence of positive cooperativity, as evaluated by isothermal titration calorimetry studies, supported by surface mutagenesis and proximity assays, resulted in higher PROTAC potency and selectivity toward individual members of the BET family proteins.

Further research demonstrated that TC formation can be more important for IPD than binary affinity of POI ligand or complete PROTAC toward the corresponding biological target. For example, foretinib-based PROTAC that possesses relatively low binary affinity toward p38α (K_d = 11 μM) can nevertheless induce the degradation of this POI (DC_{50} = 210 nM, D_{max} (maximum content of target protein degraded) = 91 %).[40] It was noticed that this PROTAC induce the TC formation *via* stabilizing PPIs between p38α and VHL

E3 ubiquitin ligase. It compensated low PROTAC binary affinity for p38α and ultimately led to POI degradation. Therefore, even low-affinity interactions between PROTACs and the corresponding POIs may be stabilized by high-affinity interactions within TC, leading to efficient POI degradation. In accordance with this finding, high affinity POI ligand is not sufficient to obtain potent PROTACs.[41]

Given that PROTACs are a part of the event-driven pharmacology (EDP), it provides additional selectivity toward homologous biological targets when compared to traditional inhibitors. The above mentioned foretinib is a promiscuous kinase ligand that can bind more than 130 kinases. The Crews laboratory demonstrated that foretinib-based PROTAC has a greater binary affinity compared to foretinib itself, because it binds to more than 50 kinases, but induces the degradation of less than 10.[40] In addition, depending on which E3 ubiquitin ligase (cereblon (CRBN) or VHL) was recruited, different degradation profiles were observed. The basis of such selectivity lies in PPIs between E3 ubiquitin ligases and POIs. This conclusion was proved by the existence of proteins that interacted with PROTAC, but remained undegraded because of unstable TCs constructed with recruited E3 ubiquitin ligases.[40] Hence, it could be concluded that simple binding of PROTAC for POI does not necessarily induce degradation. This illustrates the importance of stable TCs formation.

In contrast with this finding, using high-affinity ligands for POIs in PROTAC design is not always sufficient for a biological effect. Namely, despite incorporating high-affinity ligand for BRD4 denoted as I-BET726 (K_d = 4 nM), the PROTAC molecule which contains a lower affinity ligand, denoted as JQ1 (K_d = 100 nM), was more efficient in BRD4 degradation. Such a result is the consequence of the ability of JQ1-based PROTAC to promote TC formation *via* positive cooperativity.[41] However, there are examples of PROTACs where cooperativity is less important for POI degradation.[42,43] For example, CRBN-based potent PROTACs targeting BTK (Bruton's tyrosine kinase) (DC_{50} = 1-40 nM) and BRD4 (DC_{50} = 5–50 nM) showed very little to no cooperativity.

Phillips and Fisher described PROTACs as "programmable essential activators of ubiquitin ligase enzymes":[44] *programmable*, since they can be designed to target any POI; *essential*, since ubiquitin transfer will not occur in their absence; *activators*, since they act as recyclable catalysts that mediate the formation of a catalytically active complex (*i.e.* TC). Therefore, perceiving PROTACs as activators, more precisely as catalysts for POI degradation, gives an outline to better understand PROTAC pharmacology.

Briefly, it is important to consider TC formation during the development of PROTAC molecules. It has already been stated that reliance on binary affinities of either POI ligands incorporated in PROTACs or complete PROTACs can misguide the development of potent degraders. In addition, the importance of

cooperativity in TC formation is different for individual E3 ubiquitin ligases and POIs. Hence, it is difficult to suggest some general rules at the molecular level regarding interactions within TCs. In summary, when considering PROTAC design, the main aim should be the formation of functional TCs.

S-4. INFLUENCE OF LINKERS ON PHARMACODYNAMICS OF PROTACS

The degradation efficacy of PROTACs is not only dependent on the affinity of incorporated ligands toward POIs and E3 ubiquitin ligases, but on the combination of those mentioned as well as on the properties of linkers, which ultimately leads to TC formation, POI polyubiquitination and degradation.[45,46] In fact, it is known nowadays that both the chemical composition and length of the linker have great impacts on TC formation, degradation activity and selectivity. Besides, linkers perform specific interactions inside TCs and therefore play an important role in positive cooperativity on TC formation.[39,47] These findings could be useful in the design of PROTACs that display isoform selectivity across structurally related POIs.[42] Additionally, as already stated above, linker-mediated binding cooperativity could be a source of binding affinity toward POIs for PROTACs based on low-affinity POI ligands.[47] However, the chemical compositions of linkers, particularly their lengths and attachment points within PROTACs, have to be optimized for every pair of POI and E3 ubiquitin ligase with which they interact. This should not come as a surprise if TC structural complexity and dynamics are taken into account. Hence, it is very challenging to anticipate which combination of POI/E3 ubiquitin ligase ligands and linker will induce optimal POI degradation. Since the literature does not provide any specific guidelines regarding linkers or any strategy for their design, achieving and optimization of biological activity has been performed through iterative linker modification, where PEG- or alkyl-based linkers are commonly used first.

S-5. PROTAC TECHNOLOGY ADVANTAGES

A detailed presentation of the advantages of PROTAC technology over the other pharmacological approaches can be summarized as follows:

The possibility to tackle pharmacologically intractable proteome

About 20-25 % of therapeutically relevant biological targets are susceptible to conventional drug discovery strategies. Some of those are kinases, G protein-coupled receptors, nuclear hormone receptors and ion channels.[48,49] The proteins with no catalytic activity and/or catalytic-independent functions are still considered as undruggable biological targets.[50] There are several reasons for this, but the most important is that current therapeutic modalities are unable to tackle those potentially therapeutically relevant proteins. Some biological targets that belong to undruggable proteome are transcription factors (c-Myc,[51] β-catenin[52]) and scaffold proteins (BCL10,[53] β-arrestin[54]). These POIs are attractive

biological targets and progress in this field has been made by blocking the binding of specific interaction partners of these proteins. Furthermore, aggregated proteins common in neurodegenerative disorders, such as Alzheimer's[55] or Huntington's[56] disease, are especially convenient to be degraded using PROTACs. Although some steps toward blocking PPIs using small-molecule-based inhibitors have been made, this medicinal chemistry field remains challenging since small molecules are directed to inhibit interactions occurring on large PPI surfaces.[57] As already stated, the possibility of PROTACs to interact with any suitable binding site on POIs and perform biological effects is a very prominent feature. More precisely, it is not necessary to either block catalytic functions of POIs or PPIs – any POI ligand is suitable to design a POI degrader. It means that PROTAC , *i.e.* incorporated POI ligand can interact with any part of the biological target and be operative if it induces POI polyubiquitination and subsequent proteasomal degradation. Such theoretical increase in the binding sites of POIs that are suitable for interaction with PROTACs gives the opportunity for pharmacological interventions on the proteome currently considered as pharmacologically intractable using traditional approaches. Hence, the development of new pharmacological modalities that can tackle therapeutically relevant proteins using small-molecule ligands is a priority.

The possibility to overcome pharmacoresistance in cancer

This phenomenon can be explained by the example of kinase inhibitors that have been extensively developed in recent decades.[58] Although this class of drugs has been shown to be very effective in cancer treatment, pharmacoresistant cancers can frequently be seen in patients who thus develop disease recurrence. In this case, PROTAC molecules have been shown to be useful due to the degradation of the whole target proteins. For example, the L18I PROTAC molecule, which degrades mutated BTK, can overcome a form of lymphoma resistant to ibrutinib resulting from the C481S mutation.[59]

The possibility to overcome the accumulation of biological targets

It has been observed that some drugs after binding to the corresponding biological targets can cause their accumulation, even after a short period of time, and this could be explained in two ways. First, drug binding can stabilize the protein and thus prolong its half-life.[60] Such protein stabilization was seen with HER2 inhibitor lapatinib, BRD4 inhibitor JQ1, as well as MCL-1 inhibitor A-1210477.[61–63] Second, under certain circumstances, antagonism may cause compensatory upregulation of POI at the transcriptional level. For example, AR is a transcriptional repressor of its own transcript.[64] After the repression of AR *via* inhibitors, mRNA that codes AR increases, which ultimately leads to higher AR levels and sensitization to relatively low androgen levels. Thus, all the mechanisms for upregulation of biological targets may deteriorate the efficacy of

applied inhibitor. The elimination of POIs using PROTAC molecules is expected to be very useful in the case of proteins that may become insensitive to inhibitors either by a stabilization or upregulation mechanism.[65] For example, it has been shown that in the case of BRD4, the inhibitors of which rapidly lose efficacy due to upregulation, PROTAC molecules achieve the degradation of this protein as well as its transcriptional repression.[61]

The possibility to act on biological targets altered by mutations or interactions with binding partners

The appearance of point mutations is a common mechanism of acquired pharmacoresistance. After a certain time of drugging biological target, the appearance of its mutations is inevitable. This phenomenon can be seen with anti-HIV drugs,[66] as well as with drugs inhibiting cancer-related targets such as Bcr-Abl, EGFR, ALK, BTK.[65,67] The emergence of mutations in biological targets diminishes the affinities of their inhibitors, hence limiting their efficacy. However, changes in biological targets that are not the result of mutations can also occur. Namely, the appearance of resistance to Type I Janus kinase (JAK) 2 inhibitors can be attributed to the shift from JAK2:JAK2 homodimerization toward JAK1:JAK2/Tyrosine Kinase 2 (TYK2) heterodimerization.[68] Due to the formation of heterodimers, both JAK1 and TYK2 can perform the phosphorylation of JAK2, thus restarting downstream signaling even in the presence of JAK2 inhibitors. Induced protein degradation using PROTAC molecules is able to tackle these resistance mechanisms, since PROTAC molecules can degrade mutated biological targets. On the other hand, the degradation of therapeutically relevant proteins would prevent inhibitor-induced interactions with auxiliary proteins, which, as already stated, result in biological targets resistant to the applied inhibitors.[65]

Selectivity advancement

The ideal small-molecule antagonist inhibits only pathogenic proteins and does not act on the rest of the proteome. Often, the therapeutic index is limited by the difference in the drug potencies toward the disease-related protein and its wild-type variety. In general, selectivity toward disease-causing proteins (*e.g.* mutated ones) is difficult to achieve and only a few drugs are more active against them compared to the physiological forms of the proteins.[65] For example, dabrafenib has a higher affinity toward mutated BRAF-V600E protein associated with melanoma than for the non-mutated one.[69] Performing pharmacological effects on mutated proteins over closely related wild-type ones is very demanding. It could be assumed that PROTAC technology is a unique opportunity to improve the selectivity of therapeutics. Namely, PROTACs lead to the IPD through two steps: first, the PROTAC molecule binds to the biological target, and then E3 ubiquitin ligase transfers ubiquitin to the exposed lysine residue of the biological target. While the first step is limited by the ability to

design a selective ligand for POI, the second step, depending on the position of the exposed lysine relative to the E3 ubiquitin ligase, could be modulated to achieve selectivity even among analogous proteins.[65] For example, the JQ1-based PROTAC molecule, which binds both BRD2 and BRD4 while recruiting VHL, has been shown to have about 10-fold greater potency in BRD4 degradation, although JQ1 has almost the same affinities toward BRD2 and BRD4.[61,70] Thus, a POI bound to a PROTAC molecule does not necessarily have to be degraded. Protein degradation can only occur after its polyubiquitination and this fact should be kept in mind when considering the activity and selectivity of PROTAC molecules. In other words, different proteins with very similar catalytic sites may differ both in amino acid sequences and spatial conformations. Thus, differences within catalytic sites are not necessary to exist, but outside of them they can be very significant. This observation should be perceived as weak points of therapeutically relevant proteins to be exploited using PROTAC molecules.[65]

PROTACs' catalytic MOA

Molecules performing catalytic MOA can be applied at sub-stoichiometric amounts compared to their biological targets, resulting in smaller amounts of such molecules being required to achieve the appropriate pharmacological effects. The fact that has already been stated before is that as long as the POI ligands within the PROTAC molecules do not covalently bind to the biological targets, these molecules perform catalytic MOA.[71] The catalytic nature of PROTAC molecules is manifested by their drastic increase in potency. For example, comparative studies of a PROTAC molecule and a corresponding inhibitor have shown a more significant effect of the former on cell proliferation and apoptosis initiation compared to the latter.[61] Thus, it could be concluded that PROTAC molecules can achieve and maintain the degradation of biological targets even in small quantities. In addition, the ability of these molecules to achieve an appropriate level of protein degradation at relatively low exposures may provide a better therapeutic index since the potential to perform effects outside the desired biological targets is reduced, and thus the toxicity.[65]

EDP framework and prolonged biological effect

When small-molecule, non-covalent inhibitors are no longer present at the site of action, signaling pathways are being restored and thus, the efficacy of these types of drugs is being compromised. On the other hand, the formation of covalent bonds between covalent inhibitors and biological targets may produce pharmacological effects that, in pharmacokinetic terms, exceed exposure to the inhibitors.[72] This phenomenon can be illustrated by the example of ibrutinib. Namely, this drug covalently binds to the cysteine in the active site of BTK. With a dosing regimen of 560 mg once daily, the drug could not be found in plasma

within 24 h, but almost complete BTK occupancy was shown at that timepoint.[73] Due to their ability to induce protein degradation, PROTAC molecules may perform a similar pharmacological pattern as covalent inhibitors. In other words, PROTAC molecules can degrade a certain amount of protein, so the resulting pharmacodynamic profiles of PROTACs do not have to correlate with their pharmacokinetic profiles.[74] This loss of the link between pharmacodynamics and pharmacokinetics is especially pronounced for long-lived proteins, because it takes time to resynthesize the requisite amount of them within cells. In the case of short-lived proteins (*i.e.* proteins with a high turnover rate), this advantage is lost and continuous exposure of such biological targets to PROTAC molecules is required.[65] Thus, the concept of EDP differs significantly from the concept of ODP, because within the latter, drugs must constantly inhibit (*i.e.* occupy) their corresponding targets. Thus, three positive aspects of EDP can be stated:[65] a) Exposure to EDP-derived drugs does not have to be continuously above a certain, efficacious level; b) Lower exposure to EDP-derived drugs reduces the risk of side effects and toxicity due to off-target binding, since the selectivity diminishes at high concentrations of ODP-derived drugs; c) PROTAC molecules can cause degradation of POIs even in the presence of higher affinity ligands or in the case of POI interaction with binding partners. For example, if a ligand such as ATP (in the case of kinases) or a natural hormone such as dihydrotestosterone (in the case of AR) has a high affinity for the corresponding target and the PROTAC molecule is designed to bind to the same binding site, it still has the ability to cause degradation of the target, because only its transient binding and subsequent formation of TC is required for a biological effect. In addition to this, the ability of small molecules to interfere with high-affinity PPIs requires a high affinity of such molecules for this kind of biological targets.[75] However, the transfer of ubiquitin onto POIs occurs very rapidly and, once polyubiquitinated, biological targets are intended for proteasomal degradation. If the potency of the ligand is not sufficient to interfere with high-affinity PPIs, it is possible that the incorporation of such ligand into PROTAC may, ultimately, lead to the degradation of POI performing such PPIs. Thus, the concept of EDP may utilize ligands that have not been shown to be good inhibitors on their own, but that could be utilized as ligands for biological targets within the following PROTAC molecules.[65]

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