Bioorganic & Medicinal Chemistry Letters 30 (2020) 127202

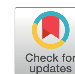
Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Digest

Monovalent protein-degraders – Insights and future perspectives



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ARTICLE INFO

Keywords:
Degradation
Monovalent
PROTAC

ABSTRACT

The therapeutic potential of interfering with dysregulated proteins by inducing its selective degradation has been pursued using different mechanisms. In the present article, we review representative examples of monovalent protein-degraders that, contrary to the proteolysis targeting chimeras, achieve target degradation without displaying recognition motifs for the recruitment of E3 ubiquitin ligases. We also highlight new technologies and assays that may brought to bear on the discovery of common elements that could predict and enable the selective degradation of pathogenic targets by monovalent protein-degraders. The successful application of these methods would pave the way to the advancement of new drugs with unique efficacy and tolerability properties.

Introduction

Dynamic modulation and post-translational modification of proteins are tightly controlled biological processes that occur in response to specific physiological responses. One such dynamic modulation is ubiquitination, which marks proteins for degradation *via* the proteasome pathway. The therapeutic potential of interfering with dysregulated proteins by inducing its selective degradation has been pursued using different drug discovery methods, most recently by proteolysis targeting chimeras (PROTACs).[1–4] These heterobifunctional molecules consist of a ligand that binds the therapeutic protein of interest and a synthon that recruits an E3 ubiquitin ligase (Fig. 1). The ternary complex formed by the targeted protein, PROTAC and E3 ligase catalyzes the removal of the pathogenic protein by the ubiquitin machinery and subsequent recovery of the PROTAC molecule to carry successive rounds of degradation. While this approach holds therapeutic promise and has attracted growing interest within the drug discovery and development communities, it is still in the early phases of maturation and only a few therapeutic targets and drugs have progressed so far into clinical trials.[5]

Unlike PROTACS, which as mentioned previously require bifunctional recognition synthons, some examples of small molecular mass compounds that bind their intended target and incidentally cause its subsequent degradation (monovalent protein-degraders; Fig. 2) have already advanced into clinical trials and, in some cases, achieved marketing approval, most notably in cancer treatment.[6] We review herein representative examples of these monovalent protein-degraders and attempt to decipher the characteristics of the ligand-protein interactions that enable the specific and effective degradation of the intended dysregulated therapeutic target. We also briefly discuss a suit of technologies and assays that could be leveraged to rationally discover and optimize monovalent protein-degraders.

Monovalent protein-degraders – representative examples

The most notable and well described monovalent protein-degraders are targeting the estrogen receptor alpha (ERα), which is a nuclear hormone protein directly implicated in the initiation and progression of hormone receptor positive solid tumors.[7]

Selective estrogen receptor modulators (SERMs) like tamoxifen (Fig. 3) effectively block the binding of estrogens to ERα, and cancer patients treated with these standard-of-care drugs often respond positively. Unfortunately, *de novo* and acquired resistance that appears in certain cancer patients ultimately leads to disease progression that may or may not respond to therapies directed to reduce the production of estrogens (e.g., aromatase inhibitors (AI) like anastrazole).[8] In these refractory or relapse disease settings, selective estrogen receptor degraders (SERDs)[9] distinguish themselves by their ability to induce the selective degradation of ERα and delay or overcome mechanisms of resistance to SERMs and AIs.[10] Given that ERα can be activated in a ligand-independent manner by signaling pathways that modulate the receptor or its associated co-factors, it is likely that the degradation of the receptor is a key contributor to the reported clinical efficacy. In this context, the only monovalent protein-degrader that has so far received marketing approval by the health authorities is fulvestrant (Fig. 3).[9] This steroid-based drug inhibits estrogen binding and causes a rapid,

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https://doi.org/10.1016/j.bmcl.2020.127202
Received 13 March 2020; Received in revised form 16 April 2020; Accepted 16 April 2020
Available online 18 April 2020
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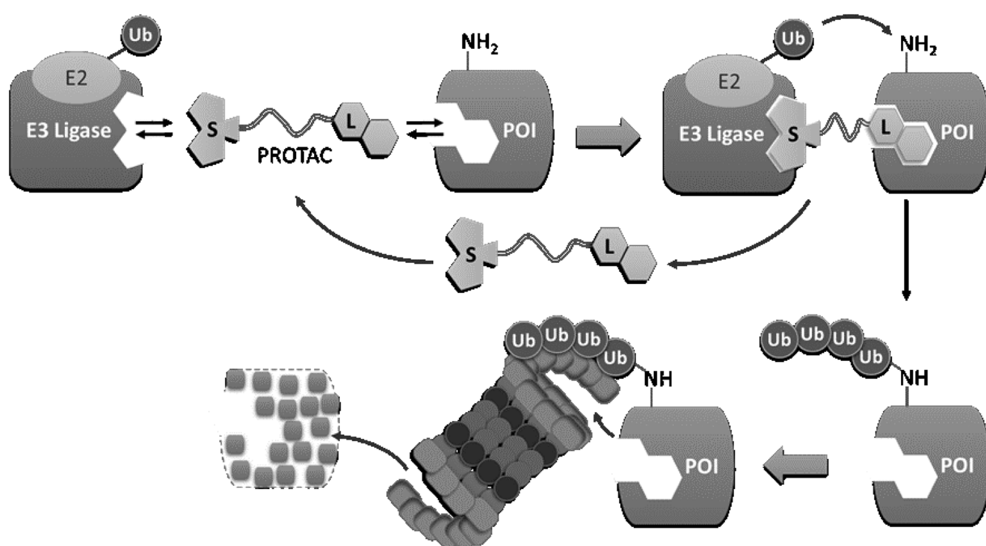

Fig. 1. Schematic representation of the mechanism of action of proteolysis targeting chimeras (PROTACs). The PROTAC molecule consists of a ligand (L) that binds the protein of interest (POI) and a synthon (S) that recruits an E3 ligase. The ternary complex catalyzes the degradation of the pathogenic protein by the ubiquitin machinery and subsequent recovery of the PROTAC molecule.

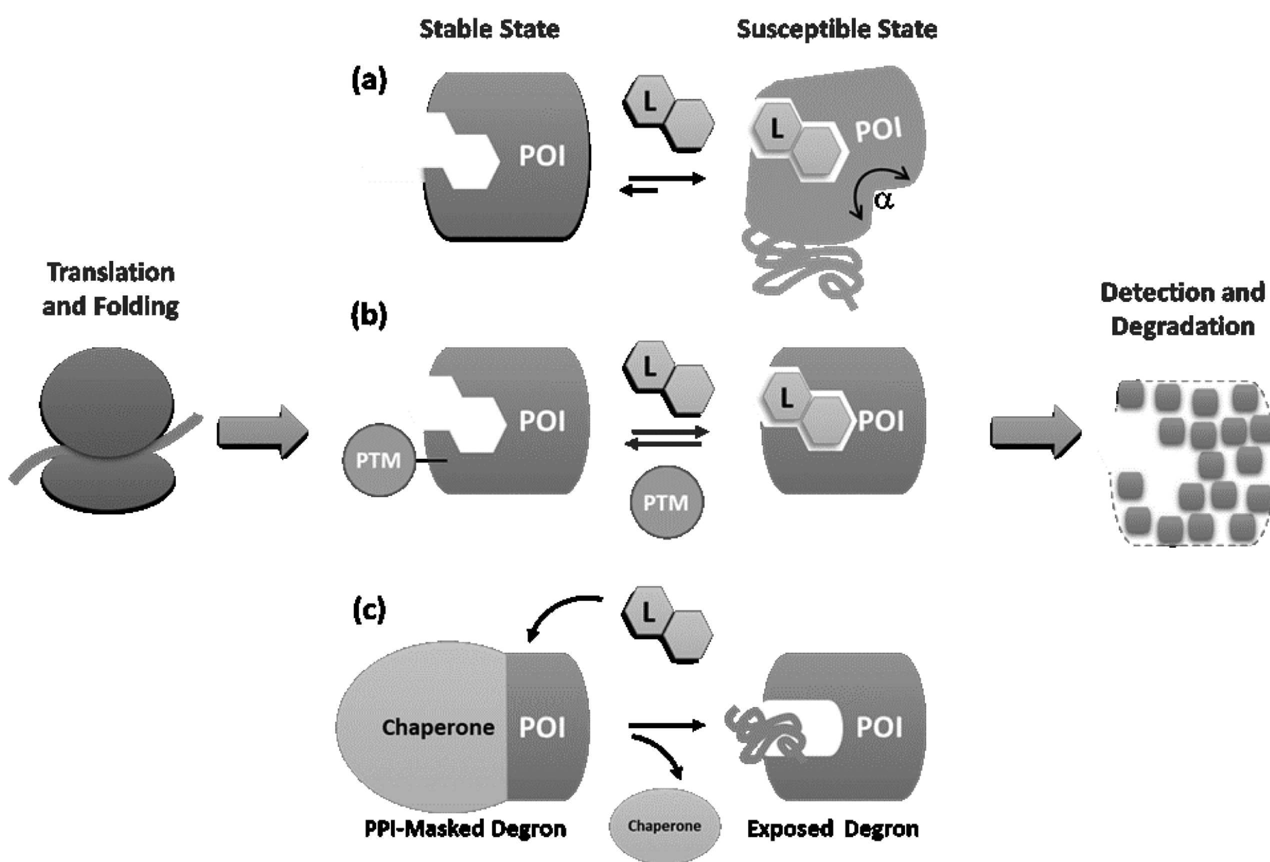

Fig. 2. Small molecular mass monovalent ligands (L) can induce the degradation of their protein of interest (POI) by producing conformational or other changes that make the protein susceptible to detection by the cellular quality control and ubiquitin-degradation machinery. The protein may enter a degradation-susceptible state for example via (a) ligand-induced conformation or folding changes, (b) affecting the post-translational modification (PTM) state of the folded POI or (c) perturbation of protein–protein interactions, including chaperone depletion.

proteasome-dependent degradation of the estrogen receptor. An unconventional long-acting intramuscular depot formulation is required to minimize its limited oral bioavailability and high pre-systemic metabolism. The drug is administered intramuscularly into the gluteal area in two 5 mL injections once monthly.[11] In spite of this demanding dosing regime, the occupancy of the receptor is not high enough to warranty durable clinical responses. It was with the preceding pharmacological shortcomings that academic institutions and pharmaceutical companies have tried to identify molecules that retained the desirable degradative properties against wild-type ERα of fulvestrant and improved its biological activity against ERα wild-type and mutants when administered orally.[12]

Most of the medicinal chemistry optimization efforts of non-steroidal SERDs (Fig. 3) have relied on cellular assays to simultaneously quantify the anti-estrogen activity (e.g., estrogen–responsive reporter gene cellular assay) and ability to degrade ERα (e.g. in-cell western

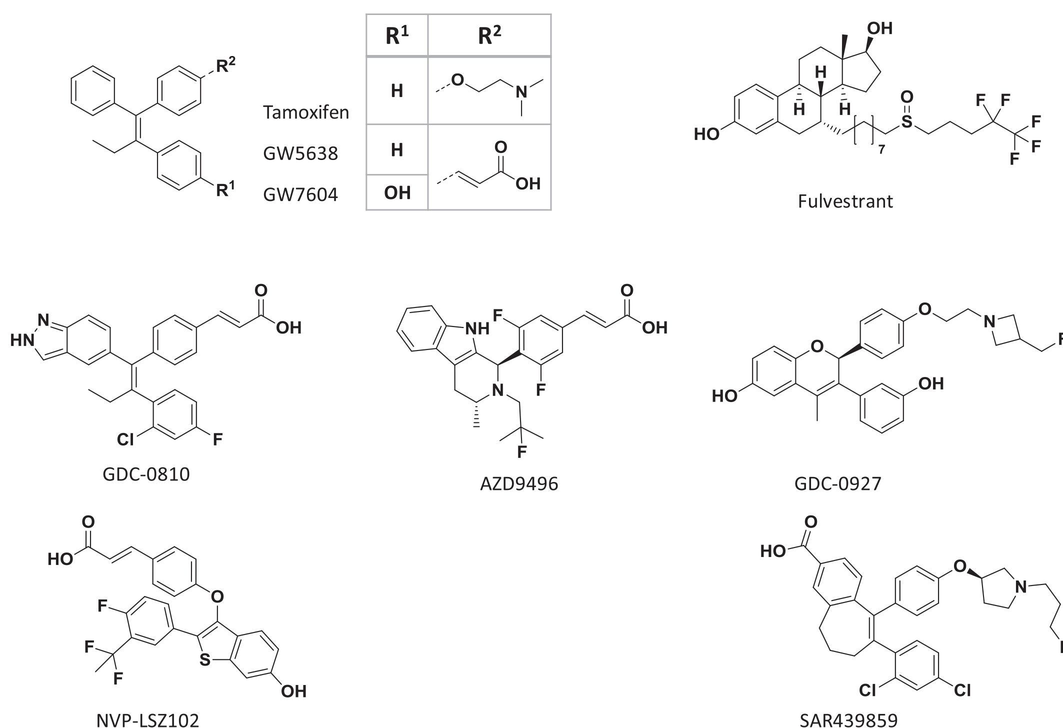

Fig. 3. Selective estrogen receptor modulators and degraders.

assay). Independently of the selected scaffold, certain common structural features associated to increase binding affinities to ERα have emerged during all these structure-activity relationship exploratory medicinal chemistry efforts,[12b] and a number of development candidates have entered clinical trials (e.g. GDC-0810, GDC-0927, AZD9496, NVP-LSZ102 and SAR439859; Fig. 3).

In parallel to the identification of new clinical candidates that could achieve more suitable biological and pharmacological profiles, the characteristics of the physical interactions that enable the specific and effective degradation of ERα by fulvestrant and non-steroidal compounds have been further investigated. Structural, biochemical and cellular studies have been performed to determine if chemically distinct SERD modulators (e.g., fulvestrant and GW5638 / GW7604; Fig. 3) were likewise functionally or mechanistically distinguishable. To this end, ERα distribution results were obtained by treating breast tumor ERα positive MCF-7 cells with estradiol and the selected SERDs. Discrete effects on receptor compartmentalization, which is a biological effect that precedes the degradation of the receptor, were observed. While the fulvestrant-occupied ERα was found to be associated with the insoluble, nuclear-associated fraction, the other compounds and estradiol were mainly in the soluble fraction. Although the observed different cellular localization properties cannot be linked to ERα dependent turnover, other findings supported the existence of mechanistically distinct modes of action for fulvestrant vis-à-vis GW5638. Elegantly conducted phage-display binding and mutagenesis studies confirmed that ERα adopts a unique structural conformation in the presence of fulvestrant, but not with GW5638.[12a] The protein-protein surface presented on ERα upon its physical contact with fulvestrant may allow its interaction with specific co-factors that target the nuclear receptor for degradation. It was speculated that the extended sulfoxide-containing alkyl side chain of fulvestrant is pushing a key structural motif (helix 12) into the co-activator binding pocket, blocking its binding but allowing the interaction with other co-factors. On the basis of the amino-acid sequence -VPNSPM- of the AEIP phage display peptide that exclusively interacts with the fulvestran-ERα complex, computational analyses were performed to determine endogenous protein that could be involved in the trafficking and stability of the receptor. Although some proteins were identified (e.g., ZMYM4 or kinesin-binding protein 1), the potential involvement of these proteins in the pharmacology of fulvestrant or other SERDS was not further confirmed. All-in-all, these early studies provided support for a link between a specific ERα conformation and stability for a sub-class of SERDS, but the structural elements required for maximal ERα degradation while maintaining antagonistic activity remained elusive.

A recent publication[13] has challenged the hypothesis that fulvestrant achieves its antagonistic effect in cancer cells through conformational destabilization and degradation of ERα. Early reports showed that even at saturated biochemical concentrations, fulvestrant and other SERDS cannot fully degrade ERα (Emax values around 85%).[12] To explain this experimental findings, it was hypothesized that low levels of ERα might not be accessible in the cell to proteolytic degradation. The residual pool of ERα may be bound to a multiprotein complex in the nuclear compartment and be slow to turn over or exported from the nucleus. A detailed head-to-head cellular evaluation of a collection of SERDS (e.g. fulvestrant, GDC-0810, GNE-274 and GDC-0927) has shown that they exhibit different transcriptional activities. Consistent with early observations that fulvestrant immobilizes ERα in the nuclear matrix (vide supra), ERα immobilization is functionally relevant for ERα transcriptional suppression and subsequent ERα turnover. Fulvestrant suppresses ERα transcriptional activity not by ERα elimination, but by markedly slowing its intra-cellular mobility. The indirect consequence of this effect is an increased in ERα turnover. This finding challenges the pre-vailing paradigm of the mechanism of action of fulvestrant and, eventually, potential strategies to identify follow-up ERα degraders. However, if this mechanism of action is translatable to other therapeutic targets, it provides a new avenue for the identification of drugs that target transcriptional factors by interfering with the assembling of dynamic signaling hubs.

Since the discovery in the early 40s that androgens promote the initiation and progression of prostate cancer, the androgen receptor has been the focus of intense drug discovery efforts. In addition to androgen deprivation therapies (e.g. abiraterone),[14] several androgen receptor antagonists (e.g. bicalutamide, enzalutamide and apalutamide; Fig. 4) have become standard-of-care drugs for the treatment of metastatic

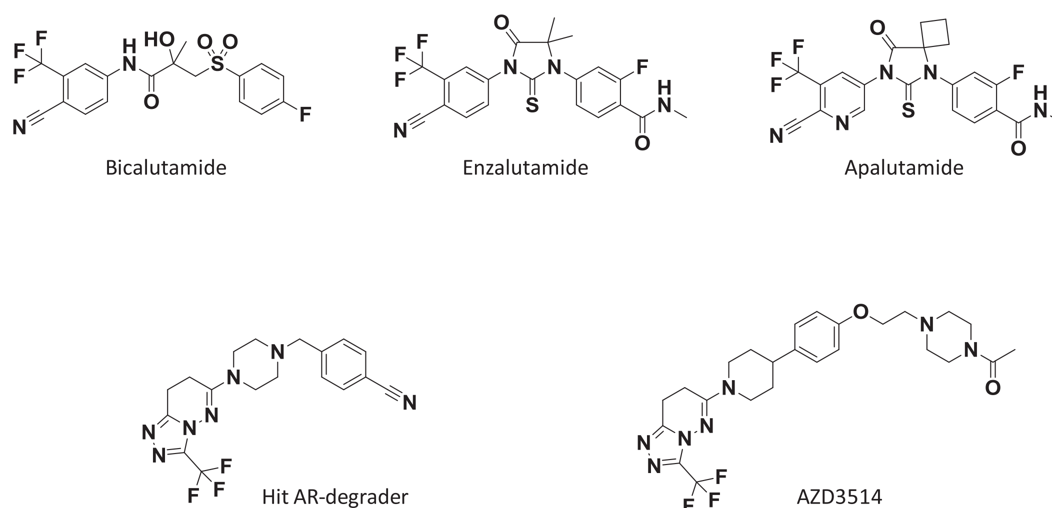

Fig. 4. Selective androgen receptor antagonists and representative androgen degraders.

prostate cancer. Although it is still controversial, it has been reported that some of these agents (e.g. bicalutamide) may decrease AR protein levels in prostate cancer cells (e.g. LNCaP and LAPPC-4) at high concentrations. However, this effect is modest and may be regulated by multiple mechanisms (e.g. interactions with the signal transducer and activator of transcription 5a/b (Stat5a/b) protein).[15]

As previously indicated for ERα, primary and acquired resistance to AR modulators are common and new strategies to block androgen receptor function in patients with metastatic castration-resistant prostate cancer have been investigated, including selective androgen receptor degraders (SARDs).[14]

A first-in-class non-steroidal SARD was identified following a medicinal chemistry optimization approach centered around a [1,2,4]triazolo[4,3-b]pyridazine chemotype. The initial hits were discovered using a high-throughput screen assay (100,000 compounds – hit rate of 1,7%) that measures affinity for the rat AR-ligand binding domain by fluorescence polarization.[16] In addition to measuring binding affinities, central to the evaluation of compounds as androgen receptor degraders was the establishment of a microtiter plate assay that determined the levels of AR in human LNCaP prostate cancer cell by immunofluorescence. Although the original lead molecule (Fig. 4) displayed appropriate pharmacokinetic properties in preclinical species, it had a potential cardiosafety red flag (pIC_{50} = 5.65 hERG assay) and limited aqueous solubility. To address the preceding pharmacological liabilities, a comprehensive medicinal chemistry optimization effort was executed. A wide exploration of chemical space by using chemical libraries and parallel synthesis combined with a structured-based drug design approach resulted in the identification of AZD3514 (binding to AR, Ki = 5 μM; AR downregulation pIC_{50} = 5.75; Fig. 4), which was selected as clinical candidate.[17] Although a reduction in total AR protein level was observed upon treatment with AZD3514 in cellular and *in vivo* efficacy studies, it was unclear if this biological effect was achieved by enhancing the rate of AR degradation and/or reducing the rate of synthesis. In this context, no effects on the rate of AR degradation were observed in the presence of proteasome inhibitors suggesting that AZD3514 treatment may affect AR levels by reducing the rate of protein synthesis (e.g., inhibition of mRNA transcription or translation). AZD3514 was evaluated in a Phase 1 trial in patients with castrate-resistant prostate cancer and although it showed encouraging results its development was discontinued due to tolerability issues.

In addition to the two nuclear receptors covered in previous sections, other therapeutic family targets have shown to be inhibited and degraded by a variety of structural distinct molecules (Fig. 5). As shown herein, the degradation of the intended dysregulated therapeutic target was often found to be fortuitous and the mechanisms associated to protein inactivation have been poorly investigated.

Structure-based designed approaches were pursued to identify potent and selective histone deacetylase 6 (HDAC6) inhibitors.[18] This HDAC isoform has been associated to neurodegenerative diseases,[19] and it is often aberrantly overexpressed in some solid tumors potentially promoting cancer cell proliferation, migration and drug resistance to chemotherapy. Cellular characterization of an optimized quinolone derivative (e.g. J22352, HDCA6 enzyme inhibition: IC_{50} = 4.7 nM – 200-fold selectivity over class 1 HDACs; Fig. 5) showed that the compound at concentrations of 1 μM and above significantly decreases the expression of its putative target in U87MG glioblastoma cancer cells. No decrease in HDAC6 abundance was observed after co-treatment with MG132, which is a well-known proteasome inhibitor, indicating the involvement of the ubiquitin degradation pathway. On the contrary, no effect was reported by co-treatment with baflomycin A1, an autophagy inhibitor that blocks the autophagosome-lysosome pathway.

HDCA6 has two catalytic domains and a *C*-terminal zinc finger ubiquitin-binding domain (ZnF-UBP) that serves as an E3 ligase binding site to recruit the ubiquitin-conjugated protein.[20] Although no experimental results were provided, the authors hypothesized that the interaction of the compound with ZnF-UBP may induce a conformational change that results in HDCA6 ubiquitination and degradation.

A surface-plasmon-resonance approach was used to enable target-based discovery of serine/threonine phosphate inhibitors.[21,22] The reported method was designed to specifically target a regulatory subunit of protein phosphatase 1 (PPP1R15B), which is a negative regulator of proteostasis. This approach allowed the identification of a new chemical entity (Raphin 1; Fig. 5) that preferentially binds R15B-PP1c (IC_{50} = 33 nM) over R15A-PP1c (IC_{50} = 977 nM). Using a variety of biochemical and biophysical methods, the authors demonstrated that the compound selectively binds to R15B, leading to an alteration of its substrate recruitment function, and consequently decreasing de-phosphorylation of eiF2alpha. Having observed that the compound had no effect on the protease sensitivity of R15A but protected R15B, the authors concluded that Raphin 1 induces a conformation change in the isolated R15B and investigated the consequences of such an event in cellular settings. In addition to inducing a transient increase of eiF2alpha phosphorylation and attenuation of protein synthesis, the compound renders R15B –and not R15A– to degradation, and effect that is blocked by co-treatment with MG132. Since R15B is reported to be a substrate of p97 and the proteasome degrades single polypeptides, the authors investigated the potential involvement of the AAA ATPase p97 in the degradation of R15B. Co-treatment with two different p97

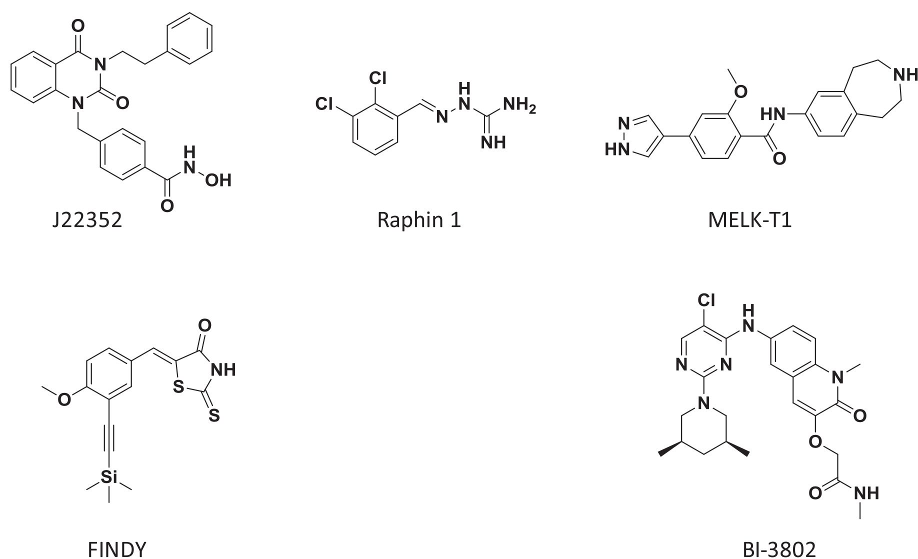

Fig. 5. Examples of monovalent protein-degraders for different therapeutic family targets.

inhibitors –MSM-873 and CB-5083– abrogated the decrease in R15B abundance caused by Raphin1.

Out of the 518 protein kinases encoded in the human genome, a selected group of tyrosine, serine/threonine and lipid kinases have been extensively investigated as therapeutic targets for the past years, and around 40 kinase modulators have received marketing approval to date.[23]

ATP- and non-competitive mechanisms of action, including compounds with reversible and irreversible kinetics, have been explored to block kinase activity, but only a limited number of examples of monovalent protein-degradation have been reported.[23a,c] One representative example is the inhibition of maternal embryonic leucine zipper kinase (MELK), which is a serine/threonine kinase that has been reported to be involved in cancer cell proliferation and DNA-damage response pathways. While the identified ATP-competitive compound (MELK-T1; Fig. 5) inhibits the kinase activity of the full-length MELK protein at sub-μM concentration (IC_{50} = 0.2 to 0.5 μM), exposure of MCF-7 to MELK-T1 at 10 μM results in a fast reduction in the endogenous MELK-1 protein level within 4 h of compound addition.[24] The fast reduction was interpreted as a signal that the compound affects the intrinsic stability of the protein and it is not interfering with the strictly controlled expression that occurs during cell-cycle progression (e.g. maximum expression during the G2/M phase and decrease at the mitotic exit). Pre-treatment of MCF-7 cells with the proteasome inhibitor MG132 for 1 h prior to the addition of MELK-T1 abolishes the reduction in cellular MELK protein. Although no supported by additional experimental data, two mechanisms were proposed to explain the unexpected cellular finding at supra-pharmacological concentrations: (i) ATP-competitive inhibition and subsequent stabilization of the ATP-like bound conformation could deprive the protein to have access to the Hsp90-Cdc37 chaperone system;[23c] and (ii) structural distortions, including interactions between sub-units like the ubiquitin-associated domain (UBA) located close the kinase domain of MELK, may alter protein folding and favor the binding to ubiquitin.

Another recent example is the inhibition of the dual-specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A), which is a potential therapeutic target in neurological disorders, cancer and diabetes, by interfering with its folding process.[25] In this case study, a cell-based assay that determines inhibition of a kinase at a transitional state during the folding process was used to identity modulators of DYRKK1A. Previous studies showed that DYRKs auto-phosphorylate first their own tyrosine residues in their transitional state and, only after maturation, they are able to phosphorylate serine or threonine residues on their substrates. To this end, isolation of transitional intermediates in a cellular setting was accomplished by selective and sequential induction of the expression the FLAG-tagged DYRK1A and TAU, a well-known substrate of DYRKK1A, fused with the destabilization domain FKBP12 (Fig. 6). Using the preceding cellular screening assay, compound FINDY (Fig. 5) was identified. The molecule blocks intramolecular autophosphorylation of residue Ser-97 in DYRK1A in cellular settings, leading to its selective degradation, but does not inhibit phosphorylation catalyzed by the mature kinase or the *in vitro* kinase activity of recombinant DYRK1A.[25] In the absence of structural information, it is unclear if the compound binds to the ATP-binding pocket or an allosteric site, but the authors demonstrated that the compound decreases the thermodynamic stability of the protein and that this mechanism is independent of the Ser-97 autophosphorylation status.

The transcription factor BCL6 has been described as an oncogenic driver of the genesis of diffuse large B cell lymphoma (DLBCL). It functions as a transcriptional repressor that binds specific DNA sequences and recruits other transcriptional co-repressor complexes. Selective blockade of these protein–protein interactions, in particular the ones involving the BTB domain of BCL6, could provide new drugs for the treatment of lymphoid malignancies. A subset of compounds that were identified using a high throughput fluorescence polarization assay (700,000 compounds) induces ubiquitination and proteasome-dependent degradation of BCL6.[26] These compounds inhibit the binding of the BTB domain of BCL6 to co-repressors, but they did not inhibit the dimerization of the BTB domain. Initial SAR studies showed that the pyridine-R^2 residue, although it is no apparently involve in any protein interaction, is critical for the monovalent protein-degradation activity: polar or charged moieties result in non-degraders, whereas non-polar or lipophilic uncharged residues at this position confer degradation activity (e.g. BI-3802; Fig. 5). Further cellular characterization of a monovalent BCL6-degrader showed antiproliferative effects of variable degrees in several DLBCL cell lines, but no significant apoptosis induction.

Other potential monovalent protein-degraders (e.g. Taselisib/p110α kinase inhibitor [27] and GNE-0011/BRD4 degrader [28] have been covered recently at several scientific events, but in the absence of peer review publications we have decided not to covered them is this article.

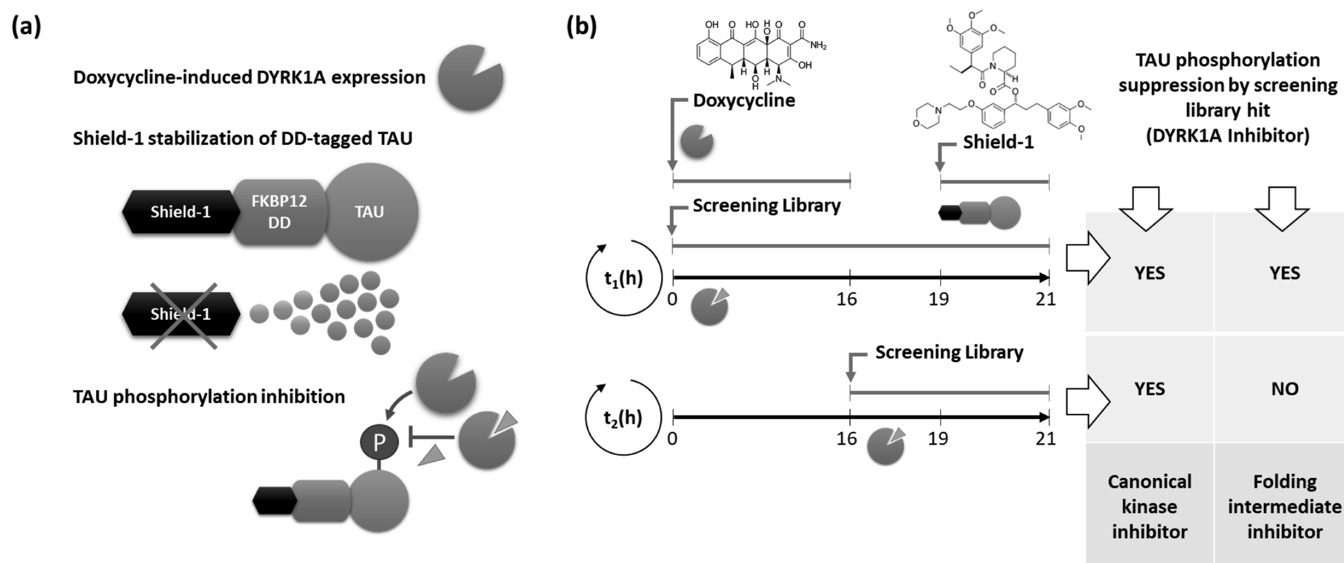

Fig. 6. Schematic diagram of the substrate phosphorylation assay by sequential induction of kinase and substrate (SPHINKS): (a) doxycycline induces DYRK1A expression; subsequently, shield-1 stabilizes TAU fused with the destabilization domain of FKPB12 (DD-TAU). DYRK1A phosphorylates DD-TA over the defined period of time (19–21 h); (b) small molecular mass molecules identified from screening were added at the indicated time points, t_1 and t_2. Canonical kinase inhibitors suppress TAU phosphorylation in both t_1 and t_2. Intermediate-selective inhibitors of DYRK1A should suppress TAU phosphorylation in t_1 but not in t_2. Graphical representation adapted from reference 25.

From opportunistic findings to the rational discovery of monovalent protein-degraders

As shown in the previous sections, the discovery of monovalent molecules that directly mediate the degradation of a dysregulated protein has been a fortuitous result prompted by the observation of fast reduction in endogenous protein levels independent of basal homeostasis. In the limited number of reported cases, the characterization of the mechanisms associated to protein degradation thwart the possibility to categorize specific elements that may enable the systematic identification of monovalent protein-degraders.

In order to increase the probability to rationally discover and optimize monovalent protein-degraders, a combination of assays with adequate throughput must be leveraged to accurately measure changes in protein binding affinities, conformation, folding and degradation rates upon compound treatment. It is anticipated that all of the above will be needed to accurately elucidate the so far elusive attributes required for optimal inactivation of a therapeutic target.

Given their sensitivity and adequate throughput to assess changes in protein conformation or folding upon compound binding, biophysical binding assays like Surface Plasmon Resonance (SPR) are broadly used for lead finding and structural-activity relationship (SAR) optimization. In addition to the widespread use of SPR for measuring protein–ligand binding events, including association and dissociation reaction kinetics, Differential Scanning Fluorimetry (DSF)[29] is a rapid and inexpensive screening method to identify small-molecular mass ligands that bind and stabilize purified proteins undergoing a temperature gradient. The difference in temperature at which the unfolding transition midpoint occurs, in the presence and absence of ligands, is related to their binding affinity and is measured by an increase in the fluorescence of SYPRO, which is a non-covalent dye with affinity for hydrophobic patches exposed upon unfolding.[30] For example, DSF was used to characterize interactions between covalent inhibitors of EGFR and Tribbles 2 pseudokinase, and the structural changes that affect its degradation in cancer cells.[31] The binding of molecules and variations in stabilization of proteins against thermal denaturation can also be measured by Differential Scanning Calorimetry (DSC). Despite the need for a reporter dye, DSF is generally favored over DSC because its shorter sampling time per compound allows a greater screening throughput.

Optical detection methods, such as Differential Scanning Light Scattering (DSLS) or Second-Harmonic Generation (SHG) polarization microscopy,[32] can also be used to measure conformational changes in physiological solutions at room temperature without labelling, thus offering an alternative for structural analysis of proteins that might not be amenable to DSF.

Hydrogen deuterium exchange mass spectrometry (HDX-MS) can also enable MoA studies to probe for regions in a protein affected by compound binding.[33] The assay measures the relative rate of deuterium incorporation, from deuterated water (D_2O), replacing amide hydrogen atoms in the backbone of a protein. As the protein incorporates more deuterium atoms, its molecular mass increases correspondingly, and this change can be measured by mass spectrometry and analyzed at the peptide level. The binding of a ligand prior to the isotopic exchange elicits a decrease in deuterium incorporation, or alterations to the hydrogen bonding network that can be relatively quantified *vs.* the untreated deuterated sample. For example, the use of HDX-MS to investigate the difference in BCL6 stabilization between two structurally similar compounds showed a slight decrease in deuterium incorporation for degrader BI-3802[26] (Fig. 5) in two regions, one close to the binding site and a distal one, suggesting a distinct MoA that could involve inducible BCL6 aggregation via dimer-dimer interaction promotion, as precedented for other BTB domain-containing proteins. While HDX-MS is very sensitive and allows precise quantitation of structural changes, its throughput is limited by the handling steps necessary to produce, without errors, the labeled protein at scale and the MS run times to test different compounds at a range of concentrations to inform SAR studies. Ongoing efforts to develop microfluidics instruments promise to minimize sample amount, experimental variability and analysis time for these experiments, augmenting the throughput and opening up the opportunity to characterize the small and fast structural transitions that occur during protein folding, ligand binding, post-translational modification and catalytic turnover. In addition to speed and throughput enhancement, coupling microfluidics to HDX-MS holds the promise to expand the study of conformation dynamics to weakly structured and intrinsically disordered protein domains and regions, inaccessible in conventional experiments, and thus expand the protein degradation druggable proteome to misfolded and disordered proteins.[34,35]

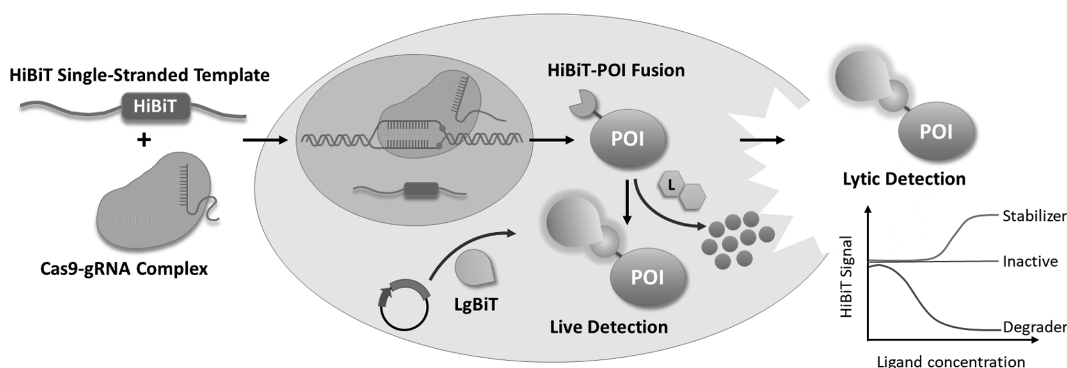

Fig. 7. Schematic diagram of HiBiT cell-based screening assay for protein abundance. CRISPR-mediated tagging of endogenous proteins with a short peptide that binds with high affinity to a larger sub-unit (LgBiT) to form a luminescent complex. Loss of signal occurs when a small molecular mass ligand (L) produces the degradation of the HiBiT tagged protein of interest (POI). Graphical representation adapted from reference 37.

Fig. 8. Multiplexed proteome dynamics profiling (mPDP) workflow. Each experiment collects samples during an eleven-point time course. mPDP combines pulse stable isotope labelling by/with amino acids in cell culture (SILAC) protein labeling with tandem isobaric mass tagging (TMT) for multiplesed dynamic analysis of proteome-wide degradation and synthesis measurements. Pulsed SILAC-TMT mPDP allows to measure in a single experiment the turnover rates for thousands of proteins expressed in a cell line. Graphical representation adapted from reference 38.

Biophysical assays that measure binding and changes in protein conformation are commonly enabled by purified recombinant protein. However, the use of truncated forms or proteins that may not present their natural post-translational modifications could cause significant limitations to investigate disease-relevant protein degradation events. For this reason, it is paramount to complement the information and throughput provided by biophysical methods with degradation studies conducted in cells, where the protein of interest is present with other interacting partners, and the in-cell protein quality control and degradation machineries are active in their physiological media.

Analysis of protein stability in eukaryotic cells has been conventionally enabled by blocking *de novo* protein synthesis with

cycloheximide (CHX), a well-known inhibitor of protein biosynthesis that prevents translational elongation and is broadly used in cell biology to determine protein half-lives.[36] Treating cells with CHX allows to determine the enhancement or decrease in protein turnover upon small molecule treatment. However, this assay is usually low in throughput and requires an antibody against the protein of interest to quantify the changes in abundance.

Among cell-based assays to study protein dynamics with sufficient scalability for high throughput screening, the luminescence-based HiBiT system (Fig. 7)[37] allows detection of low changes in protein abundance quantitatively and faster than conventional immunoassays. The HiBiT tag is a small 11 amino acid peptide that binds with high affinity to another larger subunit called LgBiT to form a complex with luciferase activity that releases a sensitive luminescent signal. Despite its small size, the tolerance for incorporation of the HiBiT tag in any protein of interest must be tested in order to confirm that the HiBiT system does not affect the function and interactome of the parent protein. It is also important to have in place appropriate counter-screening assays to eliminate false positives (luminescent interference compounds), hits with unwanted generic MoAs such as general protein translation and synthesis inhibitors, as well as to differentiate direct degraders from those that indirectly affect protein abundance.

Proteome-wide high-resolution quantitative mass spectrometry workflows allow to measure time- and perturbation-dependent protein abundance and turnover in cells, as well as understand the selectivity of the degradation events fostered by compound treatment. Multiplexed proteome dynamics profiling (mPDP; Fig. 8)[38] combines stable isotope labeling of amino acids in cell culture (SILAC) with isobaric tandem mass peptide tagging (TMT) to enable a multiplexed analysis of protein degradation and synthesis at whole cellular proteome level with mass spectrometry quantitative readout. The method is low throughput but, as exemplified by the comparative study between bromodomain inhibitor JQ1 and a JQ1-VHL PROTAC,[38] it is sensitive enough to discern proteomic differences between distinct modes of action of proteolysis targeting chimeras and corresponding protein inhibitors. It is anticipated that mPDP will also reveal differential changes for monovalent degraders as well, for it allows to classify proteins based on degradation pathways, synthesis and degradation rates, as well as their relative abundances in different cell types. In addition, such global proteome analysis allows for the quantitative assessment of compound selectivity and in-cell target engagement, providing comprehensive information on drug action.

Conclusions

In spite of the progress made over the past few years, the field of controlled degradation for targeted protein inactivation is still on the steepest part of the learning curve. As shown in the first part of this article, most of the current monomeric protein-degraders were found serendipitously in drug discovery campaigns focused on the identification of modulators of the biological function of the selected dysregulated therapeutic target. Moreover, the mechanism-of-action of some of these monomeric protein-degraders is still controversial or ill-defined.

Considering the rapid advancement of new biophysical assays and structural methods, it is forthcoming that these drug discovery technologies will be routinely leveraged to discover and rationally optimize monomeric protein-degraders, alongside with other approaches for temporary controlled protein inactivation (e.g. hydrophobic tagging,[39] autophagosome-tethering compounds,[40] Zinc finger-degrons,[41] or non-chimeric molecular glues;[42] for a recent review, see[43]). The successful application of these protein inactivation methods should expedite the advancement of tool compounds and drugs with unique biological, efficacy and tolerability properties.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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