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Proteolysis-Targeting Chimeras: Harnessing the Ubiquitin-Proteasome System to Induce Degradation of Specific Target Proteins

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Abstract

The ubiquitin-proteasome system plays a central role in regulating protein homeostasis in mammalian cells. It is a multistep process involving the poly-ubiquitination of proteins prior to their proteolytic degradation by the 26S proteasome complex. Blockade of this process results in the accumulation of proteins that are deleterious to the survival of cancer cells and has led to the approval of the proteasome inhibitors bortezomib and carfilzomib for the treatment of multiple myeloma and mantle cell lymphoma. Proteolysis-targeting chimeras (PROTACs) are bifunctional molecules designed to recruit an E3 ubiquitin ligase to a specific target protein, thereby providing a mechanism to ubiquitinate and degrade specific pathological proteins. A significant body of preclinical data, generated since PROTACs were first introduced 15 years ago, demonstrates that PROTACs provide a robust approach to expose new cell biology and to generate novel therapeutics with the potential to target currently undruggable proteins.

BACKGROUND INFORMATION

Various technologies have been developed that are designed to reduce the expression level of specific target proteins. The best established of these technologies, antisense oligonucleotides (ODN) and RNA interference (RNAi) approaches, involve the use of nucleotide-based agents. Although ODN and RNAi agents are powerful tools for basic research purposes, their development as therapeutic agents has been hindered by limited bioavailability and significant off-target activity (Rayburn & Zhang 2008). The more recent CRISPR-Cas9 gene editing technology uses direct modification of the genome to achieve gene knockout (Jinek et al. 2012, Cong et al. 2013). Although CRISPR-Cas9 is an exceptionally powerful research tool, its requirement of genetic manipulation to affect gene knockdown may limit its usefulness in the clinic. In comparison, the proteolysis-targeting chimera (PROTAC) technology is a targeted protein degradation approach that is mediated by small molecules. In contrast to the other three approaches, PROTACs degrade proteins at the posttranslational level, thereby avoiding issues related to protein stability. Importantly, the most advanced PROTACs are highly cell permeable, a feature that is important for the development of therapeutic agents. This review focuses on the use of PROTACs as a mechanism for hijacking the cellular ubiquitin-proteasome pathway (UPS) to selectively degrade target proteins both in vitro and in vivo.

The UPS is the primary mechanism used by eukaryotic cells to regulate protein turnover. The UPS is a multistep process involving the polyubiquitination of specific proteins that functionally tags proteins for degradation by the 26S proteasome (Hochstrasser 1995, Navon & Ciechanover 2009). The polyubiquitination of proteins is regulated by the concerted action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). There is one major E1 that is used by all E3s. E1 transfers ubiquitin to an E2, which then interacts with a specific E3 partner and transfers ubiquitin to a surface lysine residue on the target protein. E3, which is typically a multiprotein complex, recruits the target protein and directs the transfer of activated ubiquitin from E2 to a lysine residue on the substrate. The process is repeated in a cyclic manner, such that a new ubiquitin moiety is conjugated to an internal lysine of the previously conjugated ubiquitin molecule. This polyubiquitin chain is then bound by the 26S proteasome, leading to the degradation of the tagged protein.

PEPTIDE-BASED PROTACs

PROTACs are bifunctional molecules that are designed to recruit an E3 to a specific target protein. They consist of a target protein ligand connected via a short linker to an E3 ligand, allowing PROTACs to function as bridging compounds that bring an E3 into proximity with specific cellular proteins (**Figure 1**). The juxtaposition of the E3 complex and target protein facilitates the processive transfer of ubiquitin from the E3 complex to the target protein, thereby tagging the protein for degradation via the proteasome.

The prototypical PROTAC, PROTAC-1, exploited the Skp-Cullin-F-box-containing E3 complex (SCF). PROTAC-1 consists of an SCF^{β}-binding phosphopeptide linked to ovalicin, a small-molecule covalent binder of methionine aminopeptidase-1 (MetAP-2) (Griffith et al. 1997). Notably, PROTAC-1 was shown to tether MetAP-2 to $SCF^{\beta\text{-}TRCP}$ in cell-free extracts, followed by the polyubiquitination and degradation of MetAP-2 (Sakamoto et al. 2001). These observations provided the initial proof of concept that PROTACs could be used to harness the ubiquitin-proteolysis machinery for the destruction of selected cellular proteins. The facile extension of this approach was exemplified through the development of estradiol-SCF and dihydroxytestosterone (DHT)-SCF PROTACs, with the estradiol-SCF successfully inducing the degradation

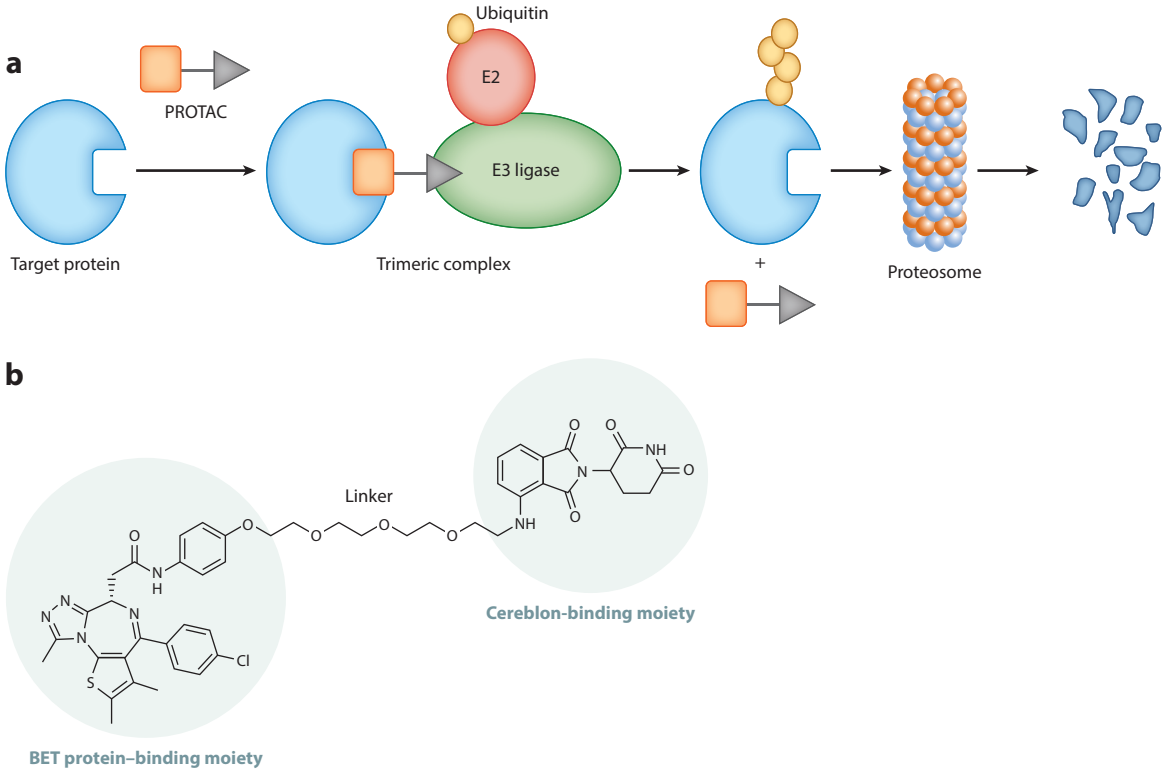

Figure 1

Proteolysis-targeting chimera (PROTAC)-mediated targeted protein degradation. (*a*) PROTACs are bifunctional molecules composed of a ubiquitin ligase (E3)–binding moiety (*gray triangle*) that is connected via a short linker to a target protein–specific ligand (*orange square*). Thus, the PROTAC functions as a bridging compound to bring an E3 complex into proximity with the target protein. The juxtaposition of the E3 complex and the target protein facilitates the processive transfer of ubiquitin from a ubiquitin-conjugating enzyme (E2) to the target protein, thereby tagging the protein for degradation via the proteasome. After adding ubiquitin to the target protein, the PROTAC is free to bind to additional target proteins. (*b*) Structure of the previously published BET protein PROTAC ARV-825 (Lu et al. 2015).

of estrogen receptor alpha (ERα) in cell-free extracts and the DHT-SCF PROTAC causing proteasome-dependent degradation of the androgen receptor (AR) following its microinjection into HEK293 cells (Sakamoto et al. 2003).

The first cell-permeable PROTACs (PROTAC-4 and PROTAC-5) capitalized on the interaction between hypoxia-inducible factor-1α (HIF-1α) and the von Hippel–Lindau E3 (VHL). VHL mediates the polyubiquitination and degradation of HIF-1α, which is a transcription factor that plays a central role in a cell's response to hypoxia (Wang et al. 1995, Ohh et al. 2000). The association of HIF-1α with VHL is mediated by its hydroxylation at proline 564 (P564) (Bruick & McKnight 2001). PROTAC-4 consists of a HIF-1α-based heptapeptide ($pVHL_7$) that spans P564 and is known to bind to VHL (Hon et al. 2002); its targeting moiety is the artificial ligand AP21998 that selectively binds the F36V mutant of FKBP12 (Rollins et al. 2000). Finally, a poly-D-arginine tag (Arg_8) was added to the carboxy-terminal portion of $pVHL_7$ to facilitate cellular uptake. Importantly, PROTAC-4 effectively degraded recombinant $FKBP12^{F36V}$-GFP protein when added to intact HeLa cells (Schneekloth et al. 2004). Likewise, PROTAC-5, which contains

the $pVHL_7$-Arg_8 peptide linked to DHT, effectively degraded the hybrid protein AR-GFP in HEK293 (Schneekloth et al. 2004). The utility of VHL-based PROTACs was further exemplified independently using MetAP-2- and ERα-targeting PROTACs that are termed small-molecule proteolysis inducers (SMPIs). Specifically, Fu-SMPI contains a HIF-1α-derived octapeptide spanning P564 ($pVHL_8$) linked to fumagillol, which is a small-molecule covalent ligand of MetAP-2 (Sin et al. 1997). Treatment of A549 lung cancer cells with Fu-SMPI resulted in time-dependent ubiquitination and degradation of MetAP-2 (Zhang et al. 2004). In the same study, $pVHL_8$-E2-SMPI caused the complete loss of ERα in intact MCF7 breast cancer cells. Importantly, a mutant form of $pVHL_8$-E2-SMPI that was unable to bind to VHL did not cause degradation of ERα, confirming that ERα degradation is dependent on the ability of the SPMI molecule to bind to both the target protein and an E3 (Zhang et al. 2004).

Subsequent studies established that PROTAC-mediated protein degradation is associated with the robust inhibition of downstream signaling cascades. Specifically, PROTACs containing a HIF-1α pentapeptide ($pVHL_5$) linked to either E2 or DHT were shown to inhibit the proliferation of hormone-dependent, but not hormone-independent, breast or prostate cancer cell lines, respectively (Rodriguez-Gonzalez et al. 2008). In addition, treatment of ERα-positive breast cancer cells with the E2-$pVHL_5$ PROTAC resulted in the decreased expression of cyclin D1 and progesterone receptor, as well as dephosphorylation of the retinoblastoma protein (Rb) and cell cycle arrest in G1 (Rodriguez-Gonzalez et al. 2008). Similarly, the DHT-VHL_5 PROTAC caused dephosphorylation of Rb and G1 cell cycle arrest in AR-positive prostate cancer cells (Rodriguez-Gonzalez et al. 2008). Notably, prolonged exposure of hormone-dependent prostate cancer cells to the DHT-$pVHL_5$ PROTAC resulted in extended growth delay and apoptosis after G1 arrest, responses that were similar to those obtained following the siRNA–mediated knockdown of AR (Linja et al. 2001, Zegarra-Moro et al. 2002, Liao et al. 2005, Eder et al. 2007). Finally, the effects of the E2-$pVHL_5$ and DHT-$pVHL_5$ PROTACs on steroid hormone signaling were blocked by the addition of competitor steroid hormone, demonstrating the specificity of the molecular response to PROTACs. These observations clearly showed that the PROTAC-driven knockdown of steroid receptors silences downstream signaling pathways that drive the proliferation of breast and prostate cancer cells.

CONDITIONAL PROTEIN DEGRADATION

All the PROTACs described above induce protein degradation regardless of the intracellular signaling context (Sakamoto et al. 2001, 2003; Schneekloth et al. 2004; Zhang et al. 2004; Rodriguez-Gonzalez et al. 2008). In contrast, two early peptide-based PROTACs coupled PROTAC-mediated degradation to the activation state of a particular signaling pathway by taking advantage of the ability of phosphotyrosine-binding (PTB) and Src homology 2 (SH2) domain–containing proteins to bind to specific phosphorylated tyrosine residues (Schlessinger & Lemmon 2003). Accordingly, two phospho-dependent PROTACs (phosphoPROTACs) were generated that linked the tyrosine phosphorylation sequences of either the nerve growth factor (NGF) receptor TrkA or the neuregulin receptor ErbB3 to $pVHL_7$ (Hines et al. 2013).

The NGF-induced dimerization of TrkA results in its autotransphosphorylation on specific tyrosine residues on its intracellular domain that create a binding site for the PTB domain–containing protein FRS2α, with FRS2α then serving as a scaffolding protein to coordinate downstream signaling (Meakin et al. 1999, Ong et al. 2000). The phosphoPROTAC $^{TrkA}PP_{FRS2\alpha}$ contained a TrkA-derived decapeptide sequence linked to the $pVHL_7$-Arg_8 peptide. Importantly, the central tyrosine of the TrkA-derived decapeptide is phosphorylated by TrkA in an NGF-dependent manner (Meakin et al. 1999). Treatment of PC12 cells with $^{TrkA}PP_{FRS2\alpha}$ resulted in NGF-dependent

phosphorylation of $^{TrkA}PP_{FRS2\alpha}$, followed by a concomitant decrease in FRS2α protein levels. Concurrent with FRS2α knockdown, $^{TrkA}PP_{FRS2\alpha}$ inhibited NGF-induced Erk1/2 activation and PC12 neuronal differentiation (Hines et al. 2013). Importantly, $^{TrkA}PP_{FRS2\alpha}$ only caused FRS2α degradation following the activation of TrkA, but not of EGFR and IGF-1R, despite the fact that all three receptors activate the same downstream signaling cascade. The latter observation underscores the remarkable specificity of phosphoPROTACs.

Neuregulin binding to ErbB3 induces the formation of ErbB2:ErbB3 heterodimers, followed by the transphosphorylation of ErbB3 and the recruitment of PI3K to ErbB3 via an SH2 domain on its p85 regulatory subunit (Hellyer et al. 2001). The phosphoPROTAC $^{ErbB3}PP_{PI3K}$ contained a 24–amino acid sequence derived from the PI3K-binding domain of ErbB3 linked to $pVHL_7$-Arg_8 (Hines et al. 2013). Treatment of MCF-7 cells with $^{ErbB3}PP_{PI3K}$ resulted in a neuregulin-dependent decrease in p85 levels, decreased Akt1 activation, and the loss of cell viability, phenotypes that are consistent with an effective block of PI3K signaling (Hellyer et al. 2001). Notably, daily subcutaneous administration of $^{ErbB3}PP_{PI3K}$ to athymic mice bearing OVCAR8 tumor xenografts resulted in a statistically significant decrease in tumor growth compared to control mice treated with either vehicle alone or a mutant version of $^{ErbB3}PP_{PI3K}$ that was unable to bind p85 PI3K (Hines et al. 2013).

$^{TrkA}PP_{FRS2\alpha}$ and $^{ErbB3}PP_{PI3K}$ represented a significant milestone in the development of PROTACs because they demonstrated the feasibility of achieving the conditional degradation of targeted proteins based on the activation state of specific signaling pathways, a degree of specificity that is not possible with other approaches such as the nucleotide-based or CRISPR technologies. Although the peptidic nature of phosphoPROTACs will likely prevent their development as therapeutic agents, they highlight the potential utility of developing state-specific ligands to generate PROTACs designed to selectively inhibit cancer cells that are dependent on the activity of a specific signaling pathway.

ALL SMALL-MOLECULE-BASED PROTACs

MDM2 Protein–Based PROTACs

The high molecular weight and labile peptide bonds of the first-generation PROTACs limit their broad applicability, especially with regard to their development as novel therapeutics. However, PROTAC technology was significantly improved through the synthesis and characterization of the first all small-molecule-based PROTAC, which exploited the E3 activity of MDM2 (Piette et al. 1997). The prototype MDM2-based PROTAC targeted AR by using a highly potent and selective AR modulator (SARM) (Marhefka et al. 2004) linked to a class of imidazoline derivatives called nutlins, which bind to MDM2 and disrupt MDM2-p53 interaction (Vassilev et al. 2004). Treatment of intact HeLa cells transiently expressing AR with the SARM-nutlin PROTAC resulted in marked degradation of AR in a proteasome-dependent manner (Schneekloth et al. 2008).

IAP-Based PROTACs

A second class of fully chemical PROTACs exploited the E3 activity of the cellular inhibitor of apoptosis protein 1 (cIAP1) and was named SNIPER (specific and nongenetic IAP-dependent protein eraser) (Itoh et al. 2010, Okuhira et al. 2011). An early cIAP1-based PROTAC, SNIPER(CRABP)-2, consisted of the cIAP1-specific ligand methyl bestatin (MeBS) (Sato et al. 2008, Sekine et al. 2008) linked to all-*trans* retinoic acid (ATRA), an endogenous ligand that binds to cellular retinoic acid–binding proteins (CRABP-I and -II) (Fogh et al. 1993, Donovan

et al. 1995). Treatment of intact cells with SNIPER(CRABP)-2 resulted in the robust degradation of CRABP-I and CRABP-II in a cIAP1- and proteasome-dependent manner (Itoh et al. 2010, Okuhira et al. 2011). Consistent with previous studies implicating CRABPs in tumor cell migration (Gupta et al. 2006, 2008; Vo & Crowe 1998), SNIPER(CRABP)-2-mediated degradation of CRABP-II was associated with a dramatic reduction in the migration of human neuroblastoma cells (Itoh et al. 2010). Thus, as previously demonstrated with the SCF-, VHL-, and MDM2-based PROTACs, cIAP-1-based SNIPER molecules caused the robust degradation of target protein and the concomitant suppression of the appropriate downstream signaling pathway. Furthermore, the observation that each of the first four E3s attempted were successfully exploited to generate PROTACs suggests that a majority, if not all, of E3s can be engaged by PROTAC technology if appropriate capture ligands are available.

Immunoprecipitation studies demonstrated that SNIPER(CRABP)-2 mediated the formation of cIAP-1-SNIPER(CRABP)-2-CRABP-II ternary complexes within cells, which is a central tenet of the mechanism by which PROTACs induce the degradation of a target protein (**Figure 1**). Specifically, immunoprecipitation of a glutathione *S*-transferase (GST)-tagged BIR3 domain of cIAP1 demonstrated the coprecipitation of GST-BIR3 and CRABP-II in a SNIPER(CRABP)-2-dependent manner (Itoh et al. 2010), confirming that SNIPER(CRABP)-2 functions as a bridging compound between CRABP-II and cIAP1. Further confirmation of PROTACs' ability to drive the formation of ternary complexes was recently provided through the elucidation of the crystal structure of a VHL—PROTAC-BRD4 ternary complex (Gadd et al. 2017).

In addition to inducing the degradation of CRABP-I and CRABP-II, SNIPER(CRABP)-2 also caused the degradation of cIAP1, which is consistent with the observation that MeBS alone is known to cause autoubiquitination and the proteasome-mediated degradation of cIAP1 (Fogh et al. 1993, Donovan et al. 1995). SNIPER(CRABP)-4 was designed to circumvent this potential liability by replacing the MeBS component of SNIPER(CRABP)-2 with bestatin methyl amide based on a previous study demonstrating that bestatin methyl amide binds to cIAP1 without inducing its degradation (Sekine et al. 2008). Importantly, the treatment of cells with SNIPER(CRABP)-4 caused the sustained degradation of CRABP-II without inducing cIAP1 degradation and resulted in a more prolonged suppression of CRABP-II than that observed with SNIPER(CRABP)-2 (Okuhira et al. 2011). However, it is important to highlight the fact that subsequent amide-based SNIPERs targeting ERα (Okuhira et al. 2013) and transforming acidic coiled-coil-containing protein 3 (TACC3) (Ohoka et al. 2014) caused the concomitant degradation of both cIAP1 and their respective target proteins. Thus, the incorporation of an amide bond into a SNIPER is not a universal remedy for the MeBS-induced autoubiquitylation and degradation of cIAP1.

Although cIAP1 was shown to be the primary E3 mediating targeted protein degradation in SNIPER(CRABP)-2-, SNIPER(CRABP)-4-, and SNIPER(ER)-treated cells (Sekine et al. 2008, Itoh et al. 2010, Okuhira et al. 2013), the siRNA knockdown of cIAP1 in SNIPER(TACC3)-treated cells did not diminish the level of TACC3 protein reduction caused by SNIPER(TACC3) (Ohoka et al. 2014). Interestingly, the siRNA knockdown of the E3 APC/C^{CDH1}, which is the natural E3 that mediates TACC3 ubiquitination (Jeng et al. 2009), abrogated the SNIPER(TACC3)-mediated degradation of TACC3 (Ohoka et al. 2014). Furthermore, the knockdown of CDH1, the substrate receptor for TACC3, also abolished the SNIPER(TACC3)-mediated reduction of TACC3, whereas knockdown of CD20, another substrate receptor in APC/C for other proteins (Peters 2006), did not affect SNIPER(TACC3)-mediated TACC3 degradation. Interestingly, SNIPER(TACC3) was shown to increase the interaction between APC/C^{CDH1} and TACC3, but not with other natural substrates. Thus, in contrast to other bestatin-based SNIPER molecules that hijack cIAP1 to mediate the degradation of their target proteins, SNIPER(TACC3) upregulates the activity of a natural E3 to degrade its target protein.

The bestatin-based SNIPERs described above suffer from the fact that their low potency often requires concentrations in excess of 10 μM (Itoh et al. 2010; Okuhira et al. 2011, 2013; Ohoka et al. 2014). Therefore, alternative SNIPER(CRABP) molecules were generated in which the cIAP1-selective ligand MeBS was replaced with the pan-IAP antagonist MV1 (Varfolomeev et al. 2007) to generate SNIPER(CRABP)-6 (Itoh et al. 2012). Notably, SNIPER(CRABP)-6 is approximately ten times more potent than SNIPER(CRABP)-2 in its ability to degrade CRABP-II and demonstrated greater antiproliferative and caspase-activating activity in MycN-amplified neuroblastoma IMR-32 cells compared to SNIPER(CRAB)-2 (Itoh et al. 2012). Although SNIPER(CRABP)-6 was shown to downregulate cIAP1, it was not determined which IAP mediated the degradation of CRABP-II. However, these data did suggest that SNIPERs containing pan-IAP antagonists as the E3-capturing moiety have greater potency compared to those that selectively recruit cIAP1.

Recently, the incorporation of the potent SMAC mimetic LCL-161 (Fulda & Vucic 2012, Infante et al. 2014) as an IAP ligand into SNIPERs resulted in the development of SNIPERs with nanomolar in vitro potency against several target proteins, as well as the first SNIPER to demonstrate robust in vivo efficacy (Ohoka et al. 2017). More specifically, SNIPER(ER)-87, which consists of an LCL-161 derivative linked to 4-hydroxytomixifen (4-OHT) caused potent, rapid, and sustained degradation of ERα in breast cancer cells. Furthermore, whereas SNIPER(ER)-87 caused marked degradation of cIAP1, it caused only modest degradation of X-linked IAP (XIAP). Interestingly, although SNIPER(ER)-87 mediated the coprecipitation of ERα with both cIAP1 and XIAP, XIAP was found to be more efficiently recruited to ERα compared to cIAP1, and siRNA depletion of XIAP, but not depletion of cIAP1, significantly suppressed SNIPER(ER)-87-mediated degradation of ERα, indicating that XIAP is the primary E3 ligase responsible for ERα degradation (Ohoka et al. 2017). The involvement of IAPs in SNIPER(ER)-87 was further confirmed using SNIPER(ER)-143, which retains a high affinity for ER but was unable to bind to IAP proteins. Notably, SNIPER(ER)-143 did not crosslink ERα to IAPs or cause ERα degradation (Ohoka et al. 2017). These data provided strong evidence that ternary complex formation is essential for SNIPER(ER)-87-induced degradation of ERα. Finally, daily intraperitoneal administration of SNIPER(ER)-87 to mice resulted in significant concentrations of SNIPER(ER)-87 in the blood and the efficient knockdown of ERα in the mouse ovary and in human breast tumor xenografts (Ohoka et al. 2017). Importantly, the reduction of ERα in tumor xenografts was accompanied by the suppression of tumor growth. Thus, the use of LCL-161 as the E3-capturing moiety of SNIPER(ER)-87 represented a significant milestone in the development of SNIPERs because it provided the potency necessary for achieving targeted protein degradation in vivo and did not cause the autoubiquitination and degradation of XIAP, which, presumably, contributed significantly to the sustained reduction of ERα levels.

LCL-161 was also used to generate SNIPERs with nanomolar potency against BCR-ABL, BRD4, and PDE4, which demonstrated the facile extension of the newest generation of SNIPER molecules (Ohoka et al. 2017).

VHL-Based PROTACs

All small-molecule-based PROTACs exploiting VHL have also been developed. Because hydroxylation of HIF-1α P564 is essential for HIF-1α-VHL interaction (Buckley et al. 2012a,b; Van Molle et al. 2012), the original small-molecule inhibitors of the VHL-HIF-1α interaction were rationally designed using hydroxyproline as a starting point. The prototype small-molecule VHL ligands were used to develop PROTACs capable of degrading green fluorescent protein (GFP)-HaloTag fusions (Buckley et al. 2015). HaloTag is a modified bacterial dehalogenase that was designed for functional protein analysis through its ability to covalently bind to hexyl

chloride tags (Los et al. 2008). Treatment of intact cells with HaloPROTACs resulted in VHL- and proteasome-dependent degradation of GFP-HaloTag (Buckley et al. 2015). The most potent HaloPROTAC caused more than a 90% reduction in HaloTag protein levels and had a DC_{50} (half-maximal degradation concentration) of 19 nM, a potency value that highlighted the robust nature of these early VHL-based PROTACs. Interestingly, the HaloPROTACs demonstrated reduced degradation at higher concentrations, a hook effect phenomenon commonly observed with ternary complexes in which the presence of high concentrations of binary complexes inhibits the formation of ternary complexes (Douglass et al. 2013).

An exploration of linker length highlighted the important role that the linker region plays in PROTAC-mediated protein degradation (Buckley et al. 2015). Specifically, a HaloPROTAC with three ethylene glycol units caused the highest degree of GFP-HaloTAG degradation (~90%). In comparison, HaloPROTACs with shorter linkers caused marginal knockdown of the GFP-HaloTag protein, possibly because the shorter linker lengths were associated with negative cooperativity in binding due to sterics preventing the HaloPROTACs from simultaneously binding to both GFP-HaloTag and VHL. Whereas HaloPROTACs with four or five ethylene glycol units caused marked protein degradation, the degradation was significantly less than that caused by the HaloPROTAC with only three ethylene glycol units. Furthermore, the HaloPROTACs with four or five ethylene glycol units had significant autoinhibition at high PROTAC concentrations, whereas no autoinhibition was observed with the HaloPROTAC containing three ethylene glycol units, possibly because three ethylene glycol groups resulted in a PROTAC length that maximized positive cooperativity for the PROTAC's binding to GFP-HaloTag and VHL (Douglass et al. 2013). The elucidation of a PROTAC-mediated ternary structure between BRD4 and VHL provided the strongest evidence that PROTACs mediate the cooperative binding between the target protein and E3 (Gadd et al. 2017). Analysis of this structure revealed that the two heads of the PROTAC recruited BRD4 and VHL into productive proximity, resulting in the formation of new protein-protein interactions between the target protein and E3.

Medicinal chemistry efforts led to the identification of second-generation VHL small-molecule ligands, with the most potent ligand having a K_d value of approximately 1 μM (Crews et al. 2013). These newer VHL ligands were then used to generate PROTACs targeting ERRα (PROTAC-ERRα), RIPK2 (PROTAC-RIPK2), and BRD4 (ARV-771) proteins (Bondeson et al. 2015, Raina et al. 2016). Treatment of intact cells with PROTAC-ERRα, PROTAC-RIPK2, or ARV-771 caused dose-dependent degradation of their respective target proteins in a VHL- and proteasome-dependent manner. Notably, PROTAC-RIPK2 and ARV-771 had DC_{50} values less than 2 nM, whereas PROTAC-ERRα had a DC_{50} value of 100 nM (Bondeson et al. 2015, Raina et al. 2016). The fact that a PROTAC can have a DC_{50} value up to two orders of magnitude greater than its affinity for E3 and the target protein is consistent with PROTACs acting catalytically and functioning substoichiometrically (Bondeson et al. 2015). It is likely that the lower cellular potency of PROTAC-ERRα is due to the fact that its ligand region requires further optimization.

Immunoprecipitation studies demonstrated that PROTAC-RIPK2 mediated the formation of ternary complexes in intact cells (Bondeson et al. 2015). Specifically, VHL coprecipitated with RIPK2 in a manner dependent on the dose of PROTAC-RIPK2. Increasing amounts of VHL were coprecipitated at low doses of PROTAC-RIPK2, and complex formation was reduced at high-PROTAC-RIPK2 concentrations. This phenomenon is consistent with negative cooperativity in binding resulting from increasing concentrations of binary complexes preventing the formation of ternary complexes. Furthermore, a VHL-based in vitro ubiquitination assay demonstrated that whereas RIPK2 ubiquitination increased with increasing concentrations of PROTAC-RIPK2, a decrease in RIPK2 ubiquitination was noted at the highest PROTAC concentrations, an observation that is again consistent with the negative cooperativity hypothesis. The

in vitro ubiquitination assay was also used to confirm that PROTACs caused induced ubiquitination substoichiometrically, whereby one molecule of PROTAC induces the ubiquitination and degradation of multiple molecules of target protein. Specifically, 1.0 pmol of PROTAC-RIPK2 was shown to catalyze, on average, the ubiquitination of 2.9 pmol of RIPK2, providing evidence of the catalytic nature of PROTACs (Bondeson et al. 2015).

Proteomic analysis of cells treated with PROTAC-RIPK2 demonstrated the high specificity of PROTAC-mediated protein degradation in intact cells. Strikingly, the only proteins effectively degraded by PROTAC-RIPK2 out of the 7,000 proteins analyzed were RIPK2 and MAPKAPK3, despite the fact that the RIPK2 ligand used in PROTAC-RIPK2 was equipotent for several other kinases, such as RIPK3, ABL, and TESK (Bondeson et al. 2015). Thus, PROTACs have the potential to add a degree of selectivity that goes beyond the intrinsic specificity of the targeting ligand.

The best-studied VHL PROTACs to date target the bromodomain and extraterminal domain (BET) family of proteins, containing two bromodomains that recognize and interact with acetylated lysine residues and an extraterminal domain that is believed to largely serve a scaffolding function in recruiting diverse transcriptional regulators (Belkina & Denis 2012, Shi & Vakoc 2014). Several studies establish that the BET protein BRD4 is preferentially located at superenhancer regions, which often reside upstream of important oncogenes such as c-*myc* and *Bcl-xL* and play a key role in regulating their expression (Chapuy et al. 2013, Lovén et al. 2013). Owing to its pivotal role in modulating the expression of essential oncogenes, BRD4 has emerged as a promising therapeutic target in multiple cancer types (French et al. 2008, Delmore et al. 2011, Mertz et al. 2011, Zuber et al. 2011, Wyce et al. 2013, Asangani et al. 2014, Baratta et al. 2015). Indeed, the characterization of small-molecule BRD4 inhibitors, such as JQ1, iBET, and OTX015, has demonstrated their promising therapeutic potential in preclinical models of various cancers (Delmore et al. 2011, Mertz et al. 2011, Zuber et al. 2011, Puissant et al. 2013, Wyce et al. 2013, Asangani et al. 2014, Boi et al. 2015).

The BET PROTAC ARV-771 contains the BET inhibitor JQ1 linked to a second-generation small-molecule VHL ligand (Raina et al. 2016). ARV-771 degraded BRD2, BRD3, and BRD4 in prostate cancer cells with single-digit nanomolar potency and had an antiproliferative effect that was up to 500-fold more potent than BET inhibitors in the same cell lines. Treatment with ARV-771, but not with the BET inhibitors, was associated with a significant increase in apoptotic activity. In addition, ARV-771 treatment of VCaP prostate cancer cells resulted in lower levels of both full-length AR and its splice variant AR-V7, whereas BET inhibitors only affected the latter. This finding has potential significance for the treatment of prostate cancer because accumulating evidence suggests that AR splice variants may mediate castration resistance, in part by heterodimerization with and activation of FL-AR in an androgen-independent manner (Watson et al. 2010, Cao et al. 2014, Xu et al. 2015). Lastly, and most importantly, subcutaneous administration of ARV-771, but not oral administration of BET inhibitors, caused the suppression of AR levels, its downstream signaling, and tumor regression in a prostate cancer mouse xenograft model. Subsequently, ARV-771 demonstrated a potent and superior antileukemia effect compared to OTX-015 in reducing the tumor burden in a mouse xenograft model of secondary acute myeloid leukemia, resulting in improved survival of the mice treated with ARV-771 versus OTX015 (Saenz et al. 2017). The superior efficacy of ARV-771 compared to BET inhibitors was attributed to the ability of ARV-771 to inhibit both BET scaffolding and acetyl-lysine-binding activities rather than just the latter.

Cereblon-Based Targeted Protein Degradation

Thalidomide was approved as a sedative in 1956 but was withdrawn from the market five years later due to severe teratogenic activity (Bartlett et al. 2004). Subsequently, thalidomide and its

IMiD (immunomodulatory imide drug) derivatives were approved for the treatment of erythema nodosum leprosum and multiple myeloma (Bartlett et al. 2004). Mechanism of action studies led to the identification of cereblon (CRBN), the substrate receptor of the CUL4-RBX1-DDB1-CRBN ($CRL4^{CRBN}$) E3 complex, as the molecular target of IMiDs (Ito et al. 2010, Lopez-Girona et al. 2012). Furthermore, the binding of IMiDs to $CRL4^{CRBN}$ was shown to inhibit the ubiquitination of endogenous $CRL4^{CRBN}$ substrates (Fischer et al. 2014), to redirect the ligase to ubiquitinate, and to degrade new proteins (Gandhi et al. 2014; Krönke et al. 2014, 2015; Lu et al. 2014). Specifically, thalidomide, lenalidomide, and pomalidomide repurposed $CRL4^{CRBN}$ for the degradation of Ikaros (IKZF1) and Aiolos (IKXF3) (Gandhi et al. 2014, Krönke et al. 2014, Lu et al. 2014), whereas lenalidomide, but not thalidomide or pomalidomide, induced the degradation of CK1α (Krönke et al. 2015). In addition, the antitumor activity of the recently reported CRBN-modulator CC-885 was shown to be associated with its ability to mediate the CRBN-dependent ubiquitination and degradation of GSPT1 (Matyskiela et al. 2016). These findings highlight IMiDs as the first approved drugs whose mechanism of action is dependent on their ability to redirect the UPS to degrade novel proteins. Despite this noteworthy recognition, the repertoire of target proteins currently degraded by IMiDs is limited to Ikaros, Aiolos, CK1α, and GSPT1. However, the inclusion of IMiDs as targeting agents in the generation of PROTACs has expanded the repertoire of IMiD target proteins. Specifically, IMiD-based PROTACs targeting BET proteins (Bai et al. 2017, Lu et al. 2015, Winter et al. 2015), FKBP12 (Winter et al. 2015), and BCR-ABL (Lai et al. 2016) have been reported.

Three distinct IMiD-based BET PROTACs have been reported: dBET1 consists of JQ1 linked to thalidomide, ARV-825 consists of OTX015 linked to pomalidomide, and BETd-246 consists of BETi-211 linked to thalidomide. The treatment of intact cells demonstrated that the BET PROTACs caused rapid and robust degradation of BRD2/3/4 in a CRBN- and proteasome-dependent manner (Lu et al. 2015, Winter et al. 2015). Consistent with the striking specificity reported for PROTAC-RIPK2 (Bondeson et al. 2015), the BET PROTACs dBET1 and BETd-246 degraded only BRD2, BRD3, and BRD4 out of the 5,000–7,000 proteins analyzed in two separate proteomic studies (Winter et al. 2015, Bai et al. 2017). Furthermore, a comparison of BETd-246 and BET-211 demonstrated that the two molecules caused distinct transcriptional responses in cells, with several proliferation and survival-related genes being suppressed following BET degradation, but either unaffected or upregulated by BET inhibition (Bai et al. 2017). For example, BETd-246 caused a marked decrease in the level of the antiapoptotic protein Mcl1, whereas BETi-211 did not downregulate this protein (Bai et al. 2017). Importantly, RNAi silencing of Mcl1 enhanced the apoptotic activity of BETi-211, whereas forced expression of Mcl1 reduced the apoptotic effect of BETd-246. Finally, comparisons of BET protein degraders and inhibitors demonstrated that dBET1, ARV-825, and BETd-246 were more potent in growth inhibition and apoptosis induction in vitro compared to their corresponding BET inhibitor (Lu et al. 2015, Winter et al. 2015, Bai et al. 2017). As previously discussed for the VHL-based BET PROTAC ARV-771, the differential response of BET degraders and BET inhibitors is likely due to the ability of the BET degraders to inhibit both BET protein's scaffolding and acetyl-lysine binding activities.

Daily intraperitoneal delivery of dBET1 in mice with established human MV4-11 tumor xenografts resulted in the marked knockdown of BRD4 and significant inhibition of tumor growth and established dBET1 as the first IMiD-based PROTAC to demonstrate in vivo activity (Winter et al. 2015). More recently, BETd-260, a close analog of BETd-246 but with improved pharmacokinetic properties, was shown to effectively reduce BET protein levels in mouse xenograft tissue and to cause significant tumor growth inhibition (Bai et al. 2017). The ability of BETd-260 to suppress tumor growth in vivo following intravenous administration represented a significant

milestone in the development of PROTACs, as it is the first reported example of a PROTAC being delivered to animals using a clinically acceptable route of administration.

Our lab recently developed BCR-ABL targeting PROTACs by conjugating BCR-ABL small-molecule inhibitors (Mughal et al. 2013) to either a VHL or CRBN ligand. Interestingly, this effort succeeded in degrading both BCR-ABL and c-ABL proteins using PROTACs in which either dasatinib or bosutinib was linked to CRBN but not to VHL (Lai et al. 2016). Although the dasatinib-VHL PROTAC failed to degrade BCR-ABL, it did successfully degrade c-ABL. Importantly, all PROTACs were shown to bind and inhibit BCR-ABL and c-ABL in cells, irrespective of their degradation activity (Lai et al. 2016). In a separate but related study, a cIAP1-based SNIPER was generated that successfully degraded BCR-ABL (Demizu et al. 2016). Thus, the choice of both the targeting ligand and the recruited E3 can influence the successful development of PROTACs.

HYDROPHOBIC TAG–INDUCED PROTEIN DEGRADATION

As newly translated polypeptides are properly folded, their hydrophobic residues are buried within the protein's core (Lins & Brasseur 1995). Accordingly, because the cell views high surface hydrophobicity as a sign of an improperly folded protein, chaperone proteins bind to hydrophobic residues located on the surface of a protein and direct the protein to the UPS for elimination (Kubota 2009).

Fulvestrant is a 17β-esterdiol analog that was generated by replacing the amide moiety of the 7α side chain with other polar groups and fluorination of the terminal alkyl function (Bowler et al. 1989). Binding fulvestrant to ERα induces a conformation that leads to an increase in ERα surface hydrophobicity and, subsequently, to ERα degradation (Wu et al. 2005). Furthermore, fulvestrant-induced ERα degradation was shown to block ligand-independent activation of the receptor, which is an activity that is not affected by tamoxifen (Wittmann et al. 2007). Based on its efficacy in ERα-positive metastatic breast cancer, fulvestrant gained US Food and Drug Administration approval in 2002.

Following the successful development of fulvestrant as a therapy that mediates the degradation of ERα, our lab initiated efforts to develop a small-molecule-based technology aimed at increasing the surface hydrophobicity of target proteins to induce their degradation. The feasibility of the approach was demonstrated by fusing the small-molecule hydrophobic moiety adamantine to the HaloTag haloalkane reactive linker (Los et al. 2008), with the resulting hydrophobic tag (HyT) then used to effectively mediate the degradation of HaloTag fusion proteins in intact cells and in vivo (Neklesa et al. 2011). Subsequently, additional adamantine-based HyT molecules were generated and shown to induce the degradation of erbB3/Her3 (Xie et al. 2014, Lim et al. 2015) and AR (Gustafson et al. 2015). Furthermore, the activity of the ATP-dependent chaperone HSP70 was shown to be required for the degradation of the adamantine-tagged proteins (Neklesa et al. 2011, Xie et al. 2014, Gustafson et al. 2015), which is consistent with the mechanism by which the UPS is known to degrade misfolded proteins (Kubota 2009).

HyT molecules were also reported in which protein degradation was induced by targeting ligands linked to tert-butyloxycarbonyl–protected arginine (B_3A) (Long et al. 2012, Shi et al. 2016). Specifically, ethacrynic acid–linked B_3A induced the degradation of its target protein GST-α1 as well as GST-α1-EGFP fusion proteins and endogenous GST-π in cells and lysates. Similarly, trimethoprim-linked B_3A induced the degradation of its target, *Escherichia coli* DHFR (eDHFR), as well as eDHFR-EGFP fusion proteins. The observation that the B_3A ligands could decrease the level of their target proteins in the presence of the translation inhibitor cycloheximide confirmed that the B_3A tag induced degradation. Interestingly, B_3A-HyT-induced protein degradation

neither involved the ubiquitination of the target protein nor used an ATP-dependent chaperone to direct the tagged protein to proteasome. Instead, the B_3A ligand mediated protein degradation by binding directly to the 20S proteasome (Shi et al. 2016).

Despite the successful development of fulvestrant as a targeted protein degradation therapy, the development of additional HyT therapeutics may be problematic because HyT molecules tend to have poor pharmacokinetic properties and relatively low cellular potencies (>1 μM).

FUTURE DIRECTIONS

The encouraging data reported for PROTAC-mediated protein degradation have led to the creation of three companies focused on advancing this promising technology: Arvinas in 2013 (New Haven, Connecticut), MedSyn Biopharma in 2015 (Ann Arbor, Michigan), and C4 Therapeutics in 2016 (Cambridge, Massachusetts). In addition, larger pharmaceutical companies have also begun to make significant investments in the technology (Bus. Wire 2016, Carroll 2015, Garde 2015, Taylor 2016). Despite their great promise, the full potential of PROTACs is largely untapped. Key areas for continued development include (*a*) expanding the druggable proteome, (*b*) exploring drug specificity, and (*c*) improving pharmacokinetic properties.

Expanding the Druggable Proteome

Supplemental Material

All the PROTACs discussed in this review (see **Supplemental Table 1**) target proteins that are members of the druggable proteome in that their activity is susceptible to suppression by small-molecule inhibitors, which often achieve their activity by competing with an endogenous small molecule, e.g., ATP, for a binding site on a protein. Unfortunately, only 10–14% of human proteins are predicted to be druggable (Russ & Lampel 2005). Because of their competitive mode of action, small-molecule inhibitors require high potency, as well as high and sustained systemic exposure, which often leads to off-target toxicity and suboptimal efficacy. In contrast, the catalytic nature of PROTACs is expected to require lower drug exposure to achieve efficacy and shorter drug-exposure times, which could improve toxicity profiles. Thus, the conversion of failed small-molecule drugs into PROTACs could provide a mechanism to increase the number of approved drugs.

PROTACs could have their greatest impact on the development of molecules capable of degrading the undruggable proteome. The ability to degrade transcription factors, scaffolding proteins, and other proteins without an enzymatic function would greatly expand the druggable proteome. The recently described Tau-targeting PROTAC TH006 is an example of a PROTAC that targets an undruggable protein (Chu et al. 2016). However, the fact that it is a fully peptidyl PROTAC will likely prevent its development as a therapeutic. Employment of DNA-encoded chemical libraries coupled to selection-based screens (Kleiner et al. 2011) could provide an effective method for identifying ligands that bind to specific undruggable proteins. The modular nature of PROTACs would then allow the rapid conversion of these ligands into fully chemical protein degraders and thereby render the undruggable proteome susceptible to therapeutic intervention. Furthermore, the elucidation of a ligase-PROTAC-target ternary structure highlighted the power of using the rational design of PROTACs (Gadd et al. 2017). This approach could be especially important in the design of PROTACs using targeting ligands with weak binding affinities. Furthermore, proximity AlphaLISA assays can be developed to monitor ternary complex formation (Gadd et al. 2017). Similar assays could be used to assist in the development of PROTACs targeting the undruggable proteome.

Exploring Drug Specificity

Our lab reported that PROTAC-RIPK2 had greater degradation specificity than its promiscuous targeting ligand would have suggested (Bondeson et al. 2015). This specificity probably arose because PROTAC-RIPK2 promoted a more stable RIPK2 ternary complex than it did for other proteins that bound with similar affinity to its targeting ligand. The application of molecular modeling coupled to the use of AlphaLISA-based ternary complex assays could be used to optimize the desired specificity during PROTAC development. In fact, such an approach was recently used to generate a JQ1-based PROTAC with increased degradation activity for BRD4 compared to other BET proteins (Gadd et al. 2017). Furthermore, because our lab has observed that the use of different E3 ligases impacts PROTAC specificity (Lai et al. 2016), a more thorough exploration of this finding is warranted. Towards this end, E3-focused small-molecule screens have already identified candidate ligands that can be used as recruiting ligands (Aghajan et al. 2010, Orlicky et al. 2010, Chan et al. 2013, Maculins et al. 2016). However, this is still a largely untapped area of research, as evidenced by the fact that only 5 out of the more than 600 human E3s (Metzger et al. 2012) have reportedly been used to generate PROTACs. Thus, expanded screening efforts to identify additional targeting ligands are needed. Finally, the characterization of the expression profile of E3s could lead to the identification of tissue- or disease-specific E3s that could then be used to achieve tissue- or disease-specific protein degradation. The idea of using PROTACs to add a degree of selectivity not achievable with small-molecule inhibitors further strengthens the allure of PROTACs as potential therapeutic agents.

Improving Pharmacokinetic Properties

PROTACs are not considered to be typical small molecules because they do not conform to Lipinski's rule of five, which is a rule of thumb used to determine if a compound possesses the properties of an orally active drug in humans (Lipinski et al. 1997). In fact, none of the four PROTACs with reported in vivo activity were administered orally, and only one (BETd-620) was administered using a clinically viable route of administration (Winter et al. 2015, Raina et al. 2016, Bai et al. 2017, Ohoka et al. 2017). PROTACs' relatively large size (typically 700–1,100 Da) provides numerous sites for metabolic attack. Although the high potency that PROTACs have demonstrated in cellular assays and the marked efficacy observed in in vivo xenograft studies are encouraging, continued medicinal chemistry efforts coupled to drug metabolism and pharmacokinetic studies will be required to improve the drug-like properties of PROTACs.

DISCLOSURE STATEMENT

K.G.C. is an employee of Arvinas, LLC. C.M.C. is a consultant and shareholder in Arvinas, which provides research support to the Crews lab.

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LITERATURE CITED

Aghajan M, Jonai N, Flick K, Fu F, Luo M, et al. 2010. Chemical genetics screen for enhancers of rapamycin identifies a specific inhibitor of an SCF family E3 ubiquitin ligase. *Nat. Biotechnol.* 28:738–42

Asangani IA, Dommeti VL, Wang X, Malik R, Cieslik M, et al. 2014. Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. *Nature* 510:278–82

Bai L, Zhou B, Yang C-Y, Jai J, McEachern D, et al. 2017. Targeted degradation of BET proteins in triple-negative breast cancer. *Cancer Res.* 77:2476–87

Baratta MG, Schinzel AC, Zwang Y, Bandopadhayay P, Bowman-Colin C, et al. 2015. An in-tumor genetic screen reveals that the BET bromodomain protein, BRD4, is a potential therapeutic target in ovarian carcinoma. *PNAS* 112:232–37

Bartlett JB, Dredge K, Dalgleish AG. 2004. Timeline: the evolution of thalidomide and its IMiD derivatives as anticancer agents. *Nat. Rev. Cancer* 4:314–22

Belkina AC, Denis G. 2012. BET domain co-regulators in obesity, inflammation and cancer. *Nat. Rev. Cancer* 12:465–77

Boi M, Gaudio E, Bonetti P, Kwee I, Bernasconi E, et al. 2015. The BET bromodomain inhibitor OTX015 affects pathogenetic pathways in preclinical B-cell tumor models and synergizes with targeted drugs. *Clin. Cancer Res.* 21:1628–38

Bondeson DP, Mares A, Smith IE, Ko E, Campos S, et al. 2015. Catalytic in vivo protein knockdown by small-molecule PROTACs. *Nat. Chem. Biol.* 11:611–17

Bowler J, Lilley TJ, Pittam JD, Wakeling AE. 1989. Novel steroidal pure antiestrogens. *Steroids* 54:71–99

Bruick RK, McKnight SL. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294:1337–40

Buckley DL, Gustafson JL, Van Molle I, Roth AG, Tae HS, et al. 2012a. Small-molecule inhibitors of the interaction between the E3 ligase VHL and HIF1α. *Angew. Chem. Int. Ed.* 51:11463–67

Buckley DL, Raina K, Darricarrere N, Hines J, Gustafson JL, et al. 2015. HaloPROTACs: use of small molecule PROTACs to induce degradation of HaloTag fusion proteins. *ACS Chem. Biol.* 10:1831–37

Buckley DL, Van Molle I, Gareiss PC, Tae HS, Michel J, et al. 2012b. Targeting the von Hippel–Lindau E3 ubiquitin ligase using small molecules to disrupt the VHL/HIF-1α interaction. *J. Am. Chem. Soc.* 134:4465–68

Bus. Wire. 2016. C4 Therapeutics launches with $73 million series A financing. *Bus. Wire*, Jan. 7. **http://www.businesswire.com/news/home/20160107005259/en/C4-Therapeutics-Launches-73-Million-Series-Financing**

Cao B, Qi Y, Zhang G, Xu D, Zhan Y, et al. 2014. Androgen receptor splice variants activating the full-length receptor in mediating resistance to androgen-directed therapy. *Oncotarget* 5:1646–56

Carroll J. 2015. Genentech embraces Arvinas with $300M tie-up on protein degradation. *FierceBiotech*, Oct. 15. **http://www.fiercebiotech.com/biotech/genentech-embraces-arvinas-300m-tie-up-on-protein-degradation**

Chan CH, Morrow JK, Li CF, Gao Y, Jin G, et al. 2013. Pharmacological inactivation of Skp2 SCF ubiquitin ligase restricts cancer stem cell traits and cancer progression. *Cell* 154:556–58

Chapuy B, McKeown M, Lin C, Monti S, Roemer M, et al. 2013. Discovery and characterization of super-enhancer-associated dependencies in diffuse large B cell lymphoma. *Cancer Cell* 24:777–90

Chu TT, Gao N, Li QQ, Chen PG, Yang XF, et al. 2016. Specific knockdown of endogenous Tau protein by peptide-directed ubiquitin-proteasome degradation. *Cell Chem. Biol.* 23:453–61

Cong L, Ran FA, Cox D, Lin S, Barretto R, et al. 2013. Multiplex genome engineering using CRISPR/Cas system. *Science* 339:819–24

Crews CM, Buckley D, Ciulli A, Jorgensen W, Gareiss PC, et al. 2013. *Compounds and methods for the inhibition of VCB E3 ubiquitin ligase*. US Patent No. WO2013106646A2

Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, et al. 2011. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* 146:904–17

Demizu Y, Shibata N, Hattori T, Ohoka N, Motoi H, et al. 2016. Development of BCR-ABL degradation inducers via the conjugation of an imatinib derivative and a cIAP1 ligand. *Bioorg. Med. Chem. Lett.* 26:4865–69

Donovan M, Olofsson B, Gustafson AL, Dencker L, Eriksson U. 1995. The cellular retinoic acid binding proteins. *J. Steroid Biochem. Mol. Biol.* 53:459–65

Douglass EF, Miller CJ, Sparer G, Shapiro H, Spiegel DA. 2013. A comprehensive mathematical model for three-body binding equilibria. *J. Am. Chem. Soc.* 135:6092–99

Eder IE, Culig Z, Ramoner R, Thurnher M, Putz T, et al. 2007. Inhibition of LNCaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. *Cancer Gene Ther.* 7:997–1007

Fischer ES, Böhm K, Lydeard JR, Yang H, Stadler MB, et al. 2014. Structure of the DDB1–CRBN E3 ubiquitin ligase in complex with thalidomide. *Nature* 512:49–53

Fogh K, Voorhees JJ, Astrom A. 1993. Expression, purification, and binding properties of human cellular retinoic acid-binding protein type I and type II. *Arch. Biochem. Biophys.* 300:751–55

French CA, Ramirez CL, Kolmakova J, Hickman TT, Cameron MJ, et al. 2008. BRD-NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells. *Oncogene* 27:2237–42

Fulda S, Vucic D. 2012. Targeting IAP proteins for therapeutic intervention in cancer. *Nat. Rev. Drug Discov.* 11:109–24

Gadd MS, Testa A, Lucas X, Chan KH, Chen W, et al. 2017. Structural basis of PROTAC cooperative recognition for selective protein degradation. *Nat. Chem. Biol.* 13:514–21

Gandhi AK, Kang J, Havens CG, Conklin T, Ning Y, et al. 2014. Immunomodulatory agents lenalidomide and pomalidomide co-stimulate T cells by inducing degradation of T cell repressors Ikaros and Aiolos via modulation of the E3 ubiquitin ligase complex $CRL4^{CRBN}$. *Br. J. Haematol.* 164:811–21

Garde D. 2015. Merck wagers $434M on Arvinas and its protein-disposal system. *FierceBiotech*, April. **http://www.fiercebiotech.com/partnering/merck-wagers-434m-on-arvinas-and-its-protein-disposal-system**

Griffith EC, Su Z, Turk BE, Chen S, Chang YHH, et al. 1997. Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. *Chem. Biol.* 4:461–71

Gupta A, Kessler P, Rawwas J, Williams BRG. 2008. Regulation of CRABP-II expression by MycN in Wilms tumor. *Exp. Cell Res.* 314:3663–68

Gupta A, Williams BRG, Hanash SM, Rawwas J. 2006. Cellular retinoic acid-binding protein II is a direct transcriptional target of MycN in neuroblastoma. *Cancer Res.* 66:8100–8

Gustafson JL, Neklesa TK, Cox CS, Roth AG, Buckley DL, et al. 2015. Small-molecule-mediated degradation of the androgen receptor through hydrophobic tagging. *Angew. Chem. Int. Ed.* 54:9659–62

Hellyer NJ, Kim MS, Koland JG. 2001. Heregulin-dependent activation of phosphoinositide 3-kinase and Akt via the ErbB2/ErbB3 co-receptor. *J. Biol. Chem.* 276:42153–61

Hines J, Gough JD, Corson TW, Crews CM. 2013. Posttranslational protein knockdown coupled to receptor tyrosine kinase activation with phosphoPROTACs. *PNAS* 110:8942–47

Hochstrasser M. 1995. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr. Opin. Cell Biol.* 7:215–23

Hon W, Wilson M, Harlos K, Claridge TDW, Schofield CJ, et al. 2002. Structural basis for the recognition of hydroxyproline in HIF-1α by pVHL. *Nature* 417:975–78

Infante JR, Dees EC, Olszanski AJ, Dhruia SV, Sen S, et al. 2014. Phase I dose-escalation study of LCL161, an oral inhibitor of apoptosis proteins inhibitor, in patients with advanced solid tumors. *J. Clin. Oncol.* 32:3103–10

Ito T, Ando H, Suzuki T, Ogura T, Hotta K, et al. 2010. Identification of a primary target of thalidomide teratogenicity. *Science* 327:1345–50

Itoh Y, Ishikawa M, Kitaguchi R, Okuhira K, Naito M, Hashimoto Y. 2012. Double protein knockdown of cIAP1 and CRABP-II using a hybrid molecule consisting of ATRA and IAPs antagonist. *Bioorg. Med. Chem. Lett.* 22:4453–57

Itoh Y, Ishikawa M, Naito M, Hashimoto Y. 2010. Protein knockdown using methyl bestatin-ligand hybrid molecules: design and synthesis of inducers of ubiquitination-mediated degradation of cellular retinoic acid-binding proteins. *J. Am. Chem. Soc.* 132:5820–26

Jeng JC, Lin YM, Lin CH, Shih HM. 2009. Cdh1 controls the stability of TACC3. *Cell Cycle* 8:3529–36

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816–22

Kleiner RE, Dumelin CE, Liu DR, Stockwell BR, Kaddurah-Daouk R, et al. 2011. Small-molecule discovery from DNA-encoded chemical libraries. *Chem. Soc. Rev.* 40:5707–17

Krönke J, Fink EC, Hollenbach PW, Macbeth KJ, Hurst SN, et al. 2015. Lenalidomide induces ubiquitination and degradation of CK1α in del(5q) MDS. *Nature* 523:183–88

Krönke J, Udeshi ND, Narla A, Grauman P, Hurst SN, et al. 2014. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science* 343:301–5

Kubota H. 2009. Quality control against misfolded proteins in the cytosol: a network for cell survival. *J. Biochem.* 146:609–16

Lai AC, Toure M, Hellerschmied D, Salami J, Jaime-Figeroa S, et al. 2016. Modular PROTAC design for the degradation of oncogenic BCR-ABL. *Angew. Chem. Int. Ed.* 55:807–10

Liao X, Tang S, Thrasher JB, Griebling TL, Li B. 2005. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol. Cancer Ther.* 4:505–15

Lim SM, Xie T, Westover KD, Ficarro SB, Tae HS, et al. 2015. Development of small molecules targeting the pseudokinase Her3. *Bioorg. Med. Chem. Lett.* 25:3382–89

Linja MJ, Savinainen KJ, Saramäki OR, Tammela TL, Vessella RL, Visakorpi T. 2001. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res.* 61:3550–55

Lins L, Brasseur R. 1995. The hydrophobic effect in protein folding. *FASEB J.* 9:535–40

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development setting. *Adv. Drug Deliv. Rev.* 23:3–25

Long MJC, Gollapalli DR, Hedstrom L. 2012. Inhibitor mediated protein degradation. *Chem. Biol.* 19:629–37

Lopez-Girona A, Mendy D, Ito T, Miller K, Gandhi AK, et al. 2012. Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. *Leukemia* 26:2326–35

Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, et al. 2008. HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* 3:373–82

Lovén J, Hoke HA, Lin CY, Lau A, Orlando DA, et al. 2013. Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell* 153:320–34

Lu G, Middleton RE, Sun H, Naniong M, Christopher J, et al. 2014. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science* 343:305–9

Lu J, Qian Y, Altieri M, Dong H, Wang J, et al. 2015. Hijacking the E3 ubiquitin ligase cereblon to efficiently target BRD4. *Chem. Biol.* 22:755–63

Maculins T, Carter N, Dorval T, Hudson K, Nissink JWM, et al. 2016. A generic platform for cellular screening against ubiquitin ligases. *Sci. Rep.* 6:18940

Marhefka CA, Gao W, Chung K, Kim J, He Y, et al. 2004. Design, synthesis, and biological characterization of metabolically stable selective androgen receptor modulators. *J. Med. Chem.* 47:993–98

Matyskiela ME, Lu G, Ito T, Pagarigan B, Lu CC, et al. 2016. A novel cereblon modulator recruits GSPT1 to the CRL4CRBN ubiquitin ligase. *Nature* 535:252–57

Meakin SO, MacDonald JIS, Gryz EA, Kubu CJ, Verdi JM. 1999. The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA: a model for discriminating proliferation and differentiation. *J. Biol. Chem.* 274:9861–70

Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, et al. 2011. Targeting MYC dependence in cancer by inhibiting BET bromodomains. *PNAS* 108:16669–74

Metzger MB, Hristova VA, Weissman AM. 2012. HECT and RING finger families of E3 ubiquitin ligases at a glance. *J. Cell Sci.* 125:531–37

Mughal A, Aslam HM, Khan AMH, Saleem S, Umah R, Saleem M. 2013. Bcr-Abl tyrosine kinase inhibitors: current status. *Infect. Agents Cancer* 8:23

Navon A, Ciechanover A. 2009. The 26S proteasome: from basic mechanisms to drug targeting. *J. Biol. Chem.* 284:33713–18

Neklesa TK, Tae HS, Schneekloth AR, Stulberg MJ, Corson TW, et al. 2011. Small-molecule hydrophobic tagging-induced degradation of HaloTag fusion proteins. *Nat. Chem. Biol.* 7:538–43

Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, et al. 2000. Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel–Lindau protein. *Nat. Cell Biol.* 2:423–27

Ohoka N, Nagai K, Hattori T, Okuhira K, Shibata N, et al. 2014. Cancer cell death induced by novel small molecules degrading the TACC3 protein via the ubiquitin–proteasome pathway. *Cell Death Dis.* 5:e1513

Ohoka N, Okuhira K, Ito M, Nagai K, Shibata N, et al. 2017. In vivo knockdown of pathogenic proteins via specific and nongenetic inhibitor of apoptosis protein (IAP)-dependent protein erasers (SNIPERs). *J. Biol. Chem.* 292:4556–70

Okuhira K, Demizu Y, Hattori T, Ohoka N, Shibata N, et al. 2013. Development of hybrid small molecules that induce degradation of estrogen receptor-alpha and necrotic cell death in breast cancer cells. *Cancer Sci.* 104:1492–98

Okuhira K, Ohoka N, Sai K, Nishimaki-Mogami T, Itoh Y, et al. 2011. Specific degradation of CRABP-II via cIAP1-mediated ubiquitylation induced by hybrid molecules that crosslink cIAP1 and the target protein. *FEBS Lett.* 585:1147–52

Ong SH, Guy GR, Hadari YR, Laks S, Gotoh N, et al. 2000. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. *Mol. Cell. Biol.* 20:979–89

Orlicky S, Tang X, Neduva V, Elowe N, Brown ED, et al. 2010. An allosteric inhibitor of substrate recognition by the SCF^{Cdc4} ubiquitin ligase. *Nat. Biotechnol.* 28:733–37

Peters JM. 2006. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat. Rev. Mol. Cell. Biol.* 7:644–56

Piette J, Neel H, Marechal V. 1997. Mdm2: keeping p53 under control. *Oncogene* 15:1001–10

Puissant A, Frumm SM, Alexe G, Bassil CF, Qi J, et al. 2013. Targeting MYCN in neuroblastoma by BET bromodomain inhibition. *Cancer Discov.* 3:309–23

Raina K, Lu J, Qian Y, Altieri M, Gordon D, et al. 2016. PROTAC-induced BET protein degradation as a therapy for castration-resistant prostate cancer. *PNAS* 113:7124–29

Rayburn ER, Zhang R. 2008. Antisense, RNAi, and gene silencing strategies for therapy: Mission possible or impossible? *Drug Discov. Today* 13:513–21

Rodriguez-Gonzalez A, Cyrus K, Salcius M, Kim K, Crews CM, et al. 2008. Targeting steroid hormone receptors for ubiquitination and degradation in breast and prostate cancer. *Oncogene* 27:7201–11

Rollins CT, Rivera VM, Woolfson DN, Keenan T, Hatada M, et al. 2000. A ligand-reversible dimerization system for controlling protein–protein interactions. *PNAS* 97:7096–101

Russ AP, Lampel S. 2005. The druggable genome: an update. *Drug Discov. Today* 10:1607–10

Saenz DT, Fiskus W, Qian Y, Manshouri T, Rajapakshe K, et al. 2017. Novel BET protein proteolysis-targeting chimera exerts superior lethal activity than bromodomain inhibitor (BETi) against post-myeloproliferative neoplasm secondary (s) AML cells. *Leukemia* 31:1951–61

Sakamoto KM, Kim KB, Kumagai A, Mercurio F, Crews CM, Deshaies RJ. 2001. Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *PNAS* 98:8554–59

Sakamoto KM, Kim KB, Verma R, Mercurio F, Crews CM, et al. 2003. Development of Protacs to target cancer-promoting proteins for ubiquitination and degradation. *Mol. Cell. Proteom.* 2:1350–58

Sato S, Aoyama H, Miyachi H, Naito M, Hashimoto Y. 2008. Demonstration of direct binding of cIAP1 degradation-promoting bestatin analogs to BIR3 domain: synthesis and application of fluorescent bestatin ester analogs. *Bioorg. Med. Chem. Lett.* 18:3354–58

Schlessinger J, Lemmon MA. 2003. SH2 and PTB domains in tyrosine kinase signaling. *Sci. STKE* 2003:RE12

Schneekloth AR, Pucheault M, Tae HS, Crews CM. 2008. Targeted intracellular protein degradation induced by a small molecule: en route to chemical proteomics. *Bioorg. Med. Chem. Lett.* 18:5904–8

Schneekloth JS, Fonseca FN, Koldobskiy M, Mandal A, Deshaies R, et al. 2004. Chemical genetic control of protein levels: selective in vivo targeted degradation. *J. Am. Chem. Soc.* 126:3748–54

Sekine K, Takubo K, Kikuchi R, Nishimoto M, Kitagawa M, et al. 2008. Small molecules destabilize cIAP1 by activating auto-ubiquitylation. *J. Biol. Chem.* 283:8961–68

Shi J, Vakoc CR. 2014. The mechanisms behind the therapeutic activity of BET bromodomain inhibition. *Mol. Cell* 54:728–36

Shi Y, Long MJC, Rosenberg MM, Li S, Kobjack A, et al. 2016. Boc_3Arg-linked ligands induce degradation by localizing target proteins to the 20S proteasome. *ACS Chem. Biol.* 11:3328–37

Sin N, Meng L, Wang MQ, Wen JJ, Bornmann WG, Crews CM. 1997. The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. *PNAS* 94:6099–103

Taylor NP. 2016. Boehringer strikes deal to develop protein-degrading cancer drugs. *FierceBiotech*, July. **http://www.fiercebiotech.com/biotech/boehringer-strikes-deal-to-develop-protein-degrading-cancer-drugs**

Van Molle I, Thomann A, Buckley DL, So EC, Lang S, et al. 2012. Dissecting fragment-based lead discovery at the von Hippel-Lindau protein:hypoxia inducible factor 1α protein-protein interface. *Chem. Biol.* 19:1300–12

Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, et al. 2007. IAP antagonists induce autoubiquitination of c-IAPs, NF-κB activation, and TNFα-dependent apoptosis. *Cell* 131:669–81

Vassilev LT, Vu BT, Craves B, Carvajal D, Podlaski F, et al. 2004. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 303:844–48

Vo HP, Crowe DL. 1998. Transcriptional regulation of retinoic acid responsive genes by cellular retinoic acid binding protein-II modulates RA mediated tumor cell proliferation and invasion. *Anticancer Res.* 18:217–24

Wang GL, Jiang BH, Rue EA, Semenza GL. 1995. Hypoxia-inducible factor 1 is a basic–helix–loop–helix–PAS heterodimer regulated by cellular O_2 tension. *PNAS* 92:5510–14

Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, et al. 2010. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. *PNAS* 107:16759–65

Winter GE, Buckley DL, Paulk J, Roberts JM, Souza A, et al. 2015. Phthalimide conjugation as a strategy for in vivo target protein degradation. *Science* 348:1376–81

Wittmann BM, Sherk A, McDonnell DP. 2007. Definition of functionally important mechanistic differences among selective estrogen receptor down-regulators. *Cancer Res.* 67:9549–60

Wu YL, Yang X, Ren Z, McDonnell DP, Norris JD, et al. 2005. Structural basis for an unexpected mode of SERM-mediated ER antagonism. *Mol. Cell* 18:413–24

Wyce A, Degenhardt Y, Bai Y, Le BC, Korenchuk S, et al. 2013. Inhibition of BET bromodomain proteins as a therapeutic approach in prostate cancer. *Oncotarget* 4:2419–29

Xie T, Lim SM, Westover KD, Dodge ME, Ercan D, et al. 2014. Pharmacological targeting of the pseudokinase Her3. *Nat. Chem. Biol.* 10:1006–12

Xu D, Zhan Y, Qi Y, Cao B, Bai S, et al. 2015. Androgen receptor splice variants dimerize to transactivate target genes. *Cancer Res.* 75:3663–71

Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. 2002. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res.* 62:1008–13

Zhang D, Baek SH, Ho A, Kim K. 2004. Degradation of target protein in living cells by small-molecule proteolysis inducer. *Bioorganic Med. Chem. Lett.* 14:645–48

Zuber JJ, Shi JJ, Wang EE, Rappaport ARAR, Hermann HH, et al. 2011. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* 478:524–28