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Accelerating PROTAC drug discovery: Establishing a relationship between ubiquitination and target protein degradation

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ABSTRACT

PROTACs have emerged as a new class of drugs that can target the "undruggable" proteome by hijacking the ubiquitin proteasome system. Despite PROTACs' success, most current PROTACs interface with a limited number of E3 ligases, hindering their expansion to many challenging therapeutic uses. Currently, PROTAC drug discovery relies heavily on traditional Western blotting and reporter gene assays which are insensitive and prone to artifacts, respectively. New reliable methods to monitor true PROTAC function (i.e., ubiquitination and subsequent degradation of targets at physiological expression levels) without external tags are essential to accelerate the PROTAC discovery process and to address many unmet therapeutic areas. In this study, we developed a new high-throughput screening technology using "TUBEs" as ubiquitin-binding entities to monitor PROTAC-mediated poly-ubiquitination of native target proteins with exceptional sensitivity. As a proof of concept, targets including BRD3, Aurora A Kinase, and KRAS were used to demonstrate that ubiquitination kinetics can reliably establish the rank order potencies of PROTAC with variable ligands and linkers. PROTAC-treated cell lysates with the highest levels of endogenous target protein ubiquitination - termed "Ub_{Max}" - display excellent correlations with DC_{50} values obtained from traditional Western blots with the added benefits of being high throughput, providing improved sensitivity, and reducing technical errors.

1. Introduction

Ubiquitination is a major regulatory mechanism to maintain cellular protein homeostasis by marking proteins for proteasomal-mediated degradation [1]. Given ubiquitin's role in a variety of pathologies, the idea of targeting the Ubiquitin Proteasome System (UPS) is at the forefront of drug discovery [2]. "Event-driven" protein degradation using the cell's own UPS is a promising technology for addressing the "undruggable" proteome [3]. Targeted protein degradation (TPD) has emerged as a new paradigm and promising therapeutic option to selectively attack previously intractable drug targets using PROteolytic TArgeting Chimeras (PROTACs) [4]. PROTACs are heterobifunctional molecules with a distinct ligand that targets a specific E3 ligase which is tethered to another ligand specific for the target protein using an optimized chemical linker. A functional PROTAC induces a ternary E3-PROTAC-target complex, resulting in poly-ubiquitination and subsequent controlled protein degradation [5]. Ability to function at sub-stoichiometric levels for efficient degradation, a significant advantage over traditional small molecules.

PROTACs have been explored in multiple disease fields with focus on only few ligases like cereblon (CRBN), Von Hippel-Lindau (VHL), IAP and MDM2. Cancer targets like androgen receptor, estrogen receptor, BTK, BCL2, CDK8 and c-MET [6–11] have been successfully targeted using PROTACs. A variety of BET family (BRD2, BRD3, and BRD4)- PROTACs were designed using multiple ligases; MDM2-based BRD4 PROTAC [12], CRBN based dBET1 [13] and BETd-24-6 [14] for triple-negative breast cancer, enhanced membrane permeable dBET6 [15], and dBET57 PROTAC [16]. PROTACs for Hepatitis c virus (HCV) protease, IRAK4 and Tau [17–19] have been explored for viral, immune and neurodegenerative diseases, respectively. Currently, the PROTAC field expansion to vast undruggable proteome is hindered due to narrow focus on select E3 ligases. Lack of reliable tools to rapidly evaluate PROTACs based on

Abbreviations: PROTACs, Proteolysis targeting chimeras; TUBE, Tandem Ubiquitin Binding Entities.

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new ligases is hindering the progress. Screening platforms designed must be physiologically relevant and represent true PROTAC cellular function, i.e., PROTAC-mediated target ubiquitination and degradation.

Cellular PROTAC screening is traditionally performed using cell lines harboring reporter genes and/or Western blotting. While Western blotting is easy to perform, they are low throughput, semi-quantitative and lack sensitivity. While reporter gene assays address some of the issues, they are challenged by reporter tags having internal lysines leading to artifacts. Currently, no approaches are available that can identify true PROTAC effects such as target ubiquitination and proteasome-mediated degradation simultaneously. High affinity ubiquitin capture reagents like TUBEs [20] (tandem ubiquitin binding entities), are engineered ubiquitin binding domains (UBDs) that allow for detection of ultralow levels of polyubiquitinated proteins under native conditions with affinities as low as 1 nM. The versatility and selectivity of TUBEs makes them superior to antibodies, and they also offer chain-selectivity (-K48, -K63, or linear) [21]. High throughput assays that can report the efficacy of multiple PROTACs simultaneously by monitoring PROTAC mediated ubiquitination can help establish rank order potency and guide chemists in developing meaningful structure activity relationships (SAR) rapidly.

In the current study, we employ TUBEs as affinity capture reagents to monitor PROTAC-induced poly-ubiquitination and degradation as a measure of potency. We established and validated proof-of-concept cell-based assays in a 96-well format using PROTACS for three therapeutic targets BET family proteins, kinases, and KRAS. To our knowledge, the proposed PROTAC assays are first of its kind that can simultaneously 1) detect ubiquitination of endogenous, native protein targets, 2) evaluate the potency of PROTACs, and 3) establish a link between the UPS and protein degradation. Using these TUBE assays, we established rank order potencies between four BET family PROTACs dBET1, dBET6, BETd246 and dBET57 based on peak ubiquitination signals ("Ub_{Max}") of the target protein. TUBE assay was successful in demonstrating promiscuous kinase PROTACs efficiency to degrade Aurora Kinase A at sub-nanomolar concentrations within 1 h. A comparative study to identify changes in the ubiquitination and degradation profile of KRAS G12C PROTACs recruiting two E3 ligases (CRBN and VHL). All of the ubiquitination and degradation profiles obtained from TUBE based assays correlate well with traditional low throughput immunoblotting. Significant correlation between DC_{50} obtained from protein degradation in western blotting and Ub_{Max} values demonstrates our proposed assays can aid in high-throughput screening and drastically eliminate artifacts to overcome bottlenecks in PROTAC drug discovery.

1.1. Materials and methods

1.1.1. Chemicals & reagents

Cells used in both plate-based assays and western blotting were lysed using RIPA buffer with inhibitors like MG132, 1,10-Phenanthroline, PMSF, PIC, and PR-619 to protect poly-ubiquitinated protein signature, obtained from LifeSensors Inc. PBS and PBS-T refers to 1X Phosphate-buffered saline, with and without 1% Tween 20. Immobilon Western Chemiluminescence HRP Substrates were purchased from SIGMA. All the PROTACs were purchased from Medchem Express (MCE, see Table S1). Cells were grown in DMEM/RPMI 1640, 10% Fetal Bovine Serum (FBS), 1X penicillin, and 1X streptomycin. PROTACs were added at 1000X to 10^6 - 10^7/in 1 mL cells for optimized times at various concentrations in a 96-well sterile plate and the cells were subsequently lysed in 150 μL of lysis buffer. Bromodomain PROTACs with thalidomide and pomalidomide to recruit CRBN E3 ligase and JQ1 and BETi-211 as ligands to recruit bromodomain proteins. PROTACs selection was driven by the desire to challenge our platform's ability to differentiate efficiencies of PROTACs by their ability to ubiquitinate target proteins. Proof-of-concept study, presented here, compares the effects of PROTACs with variable linker chain lengths (dBET57 **"C2,"** dBET1 **"C4,"** dBET6 **"C8,"** and PEG linker BETd-24-6 vs non-PEG linker dBET6) on ubiquitination profiles that could provide valuable information to medicinal chemists. The promiscuous kinase PROTAC (TL12-186) designed using thalidomide as a CRBN ligand and TL-13-87 connected by a PEG linker to target multiple kinases. Two K-RAS PROTACs containing covalent MRTX ligand were designed to selectively bind to G12C mutant with pomalidomide as CRBN ligand (Compound 518) and a VHL ligand for VHL PROTAC (LC-2) (Table S1).

1.1.2. TUBEs based screening platform

PROTAC assay plates used in the study are from LifeSensors Inc. (Catalogue# PA950), coated with TUBE reagents (Fig. 1). A single PROTAC assay plate could monitor 12 different PROTACs simultaneously in an 8-point dose response to establish comparative ubiquitination and degradation profiles. Plates stored at −80 °C were brought to room temperature prior to use. Cell lysates were pretreated with urea pretreatment buffer for 15 min to disrupt any native complexes from the #PA950 assay kit. Pretreated samples were diluted to 15 μg in 100 μL volume per well using PBS-T and transferred to the assay plate. Lysates were incubated for 90 min at room temperature on an orbital shaker at 300 rpm, and the plates were washed 3 times with 200 μL PBS-T. Antibodies against BRD3 (Sigma, SAB4503199, 1:1000), AURKA (LS Bio LS-C664380, 1 μg/mL), and K-RAS (Sigma, WH0003845M1, 1 μg/mL) were diluted in 1X antibody dilution buffer, 100 μL of antibody were added to each well, and the samples were incubated at room temperature for 60 min on an orbital shaker at 300 rpm. 100 μL of 1:1000 anti-rabbit/anti-mouse IgG-HRP in 1X antibody dilution buffer were added to each well after washing with PBS-T as previously described. Plates were incubated at room temperature for 45 min at 300 rpm on an orbital shaker. Following one more 3X wash cycle as described above, 400 μL of luminol reagent and HRP substrate peroxide solution were diluted to10 mL in MΩ water, and 100 μL was then transferred to each well immediately before detection on a PerkinElmer Envision® plate reader. The chemiluminescence signal was immediately captured for 3 min at 1-min intervals.

1.2. A guide for setting up rapid TUBE plate-based assays

To successfully set up HTS screening with novel PROTACs without pre-existing knowledge, we recommend the following steps. 1. Identify a model PROTAC that can potentially demonstrate activity based on knowledge in PROTAC design or *in vitro* binding studies. 2. Perform a time course study with 2−3 doses of the model PROTAC based on affinities of the ligands selected. 3. Monitor ubiquitination and degradation profiles using plate-based assay and identify time point that demonstrates Ub_{Max}. 4. Perform a dose response at selected time point with a library of PROTACs to establish rank order potency.

2. Results & discussion

2.1. TUBE based PROTAC plate assay: Ub_{Max} to predict PROTAC efficacy

The effects of BET family PROTACs, were evaluated in Jurkat cells by monitoring BRD3 degradation in a dose response study. As previously reported, treatment of Jurkat cells with dBET1 (1 μM) resulted in significant degradation of BRD3. To identify treatment

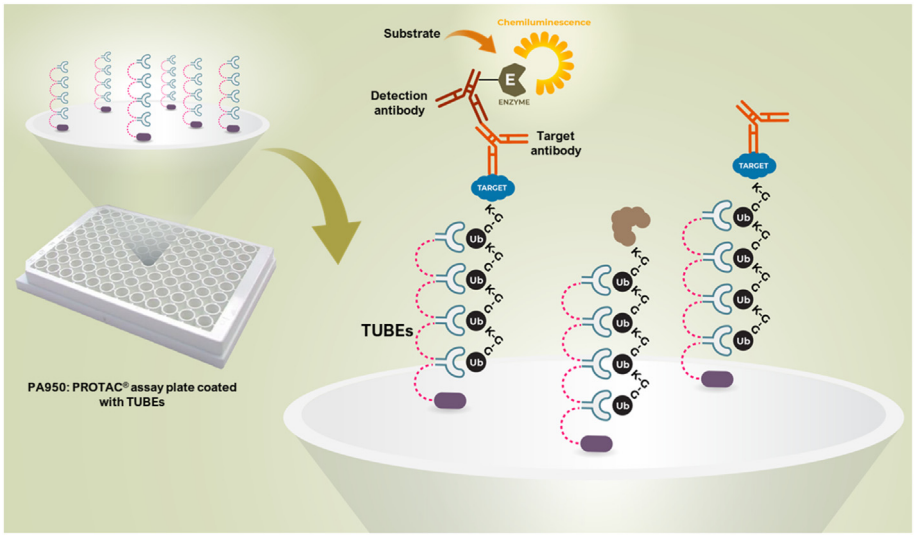

Fig. 1. Schematic representation of TUBE assay to monitor PROTAC mediated cellular ubiquitination of target proteins.

time for all 4 PROTACs in this study, we used dBET1 as the model in a time course experiment (Fig. S1 SI). We found that 45–60 min is optimal for monitoring degradation in Jurkat cells. A detailed experimental procedure of dBET1 time course study is presented in SI.

All four test PROTACs for BRD3 were treated for 45 min in an 8-point dose response (3 nM–10 μM) at 37 °C in a 96-well culture plate to study ubiquitination and establish rank order potencies. Poly-ubiquitination responses of BRD3 from PROTAC-treated cell lysates using anti-BRD3 antibody were captured as chemiluminescence (CL) signal from TUBE based assays. CL responses, demonstrated increase in CL signal with increasing concentrations reaching a maximum value (CL_{Max}), followed by a loss of CL signal representing degradation. CL_{Max}, which indicates the highest ubiquitination levels of target proteins, is referred to as the Ub_{Max}. Ub_{Max} values determined by our TUBE based platforms represents polyubiquitination of the target protein, as precursor to degradation (an intermediate phase). One of the main challenges in drug discovery involving the UPS is evaluating ubiquitin-driven degradation quantitatively and reliably to understand the kinetics and stoichiometry of rate limiting intermediates [22]. Ub_{Max} (peak ubiquitination) allows for absolute quantification of the intermediate phase of PROTAC-mediated target degradation. Relative CL intensities (Fig. 2) were calculated by dividing raw CL signals from a given PROTAC dose over DMSO treated samples. The Ub_{Max} can be readily correlated to the DC_{50}; an intermediate phase of protein degradation represented by condition at which the target is degraded by 50% [23,24]. These experiments establish Ub_{Max} as a surrogate for evaluating PROTACs efficiency and target degradability.

Maximizing efficiency of PROTAC-mediated degradation rely on productive ternary complex which brings E3 ligase in close proximity to its target protein, leading to ubiquitination. In many cases, biochemical/biophysical assays that measure this interaction fails to translate to degradation in cells as these assays does not account for role of proteins intrinsic features like exposed lysines for ubiquitination and proper structural orientation required for ubiquitination in cells. By contrast, the "Ub_{Max}" from our TUBE-based PROTAC assay platform is a direct representation of protein's ability to get and ubiquitinated via a successful PROTAC-mediated ternary complex formation followed by degradation. Thus, changes in ubiquitination can be used as a tool to establish rank order potency of PROTACs and predict their DC_{50} and D_{Max} of targets. D_{Max} is represented as maximum levels of target degradation which typically expressed as percent decrease in protein levels. In our TUBE based assays, dose at which D_{Max} is observed can be predicted based on dose where either ubiquitination signal reaches back to basal level post Ub_{Max}, referred here on as Ub_{Deg}. Ub_{Deg} is a PROTAC concentration no longer considered as rate limiting to get D_{Max} suitable for predicting potencies.

Fig. 2. displays the dose-dependent polyubiquitination of BRD3 with all four PROTACs and their Ub_{Max} values. PROTACs with varying linker lengths showed a Ub_{Max} at 0.3 μM and (D_{Max}-Ub_{Deg}) at 10 μM for C2-dBET57, 1.0 μM (Ub_{max}) and 10 μM (D_{Max} - Ub_{Deg}) for C4-dBET1 (Fig. 2A), and 0.03 μM (Ub_{Max}) and 0.1 μM (D_{Max} - Ub_{Deg}) for C8-BET6 (Fig. 2B). We observe a 10-fold increase in the potency of target protein ubiquitination by C8 linker over C2 linker. PROTAC BETd24-6, which contains a PEG linker, has Ub_{Max} of 0.03 μM, comparable to that of dBET6 and (D_{Max}-Ub_{Deg}) at 0.1 μM (Fig. 2C). The increased potency of dBET6 is consistent with literature showing dBET6 is 10 times more cell permeant than dBET1 [15], (Fig. 2B). Interestingly, dBET57 increased the ubiquitination signal over the 0.1 μM–3.0 μM concentration range (Fig. 2D), possibly resulting from the use of pomalidomide instead of thalidomide as the E3 ligand. Such crucial information of poly-ubiquitination profiles of sustained ubiquitination signals can guide medicinal chemists to rationally design potent PROTACs. Variables Ub_{Max} - DC_{50} doses under the same assay conditions indicates that the TUBE based assays designed can predict target's PROTACabiltiy and PROTAC's efficiency. Since ubiquitination is a dynamic process and the transient ubiquitination species are short lived a reasonable fold increase >1.5 – 2-fold can be considered reliable. We have compared our results with Promega reporter gene assays that function by endogenously tagging targets with split Nano-Luc reporter tag called HiBiT and HaloTag- ubiquitin to study ubiquitination. Interestingly, we have observed our proposed approach has ~ 4-fold increase in ubiquitination signal at 1 μM for native endogenous BRD3 and wildtype ubiquitin in Jurkat cells using dBET1 PROTAC compared to ~ 3-fold increase (signal-to-noise ratio) in BRET signal in HEK293 cells using Promega assays [25].

Traditional Western blot analysis of whole cell lysates derived from cells treated with PROTACs display successful BRD3 degradation and subsequent rescue with the proteasome inhibitor, MG132, within a 45-min time interval (Fig. 3). Some of the PROTACs

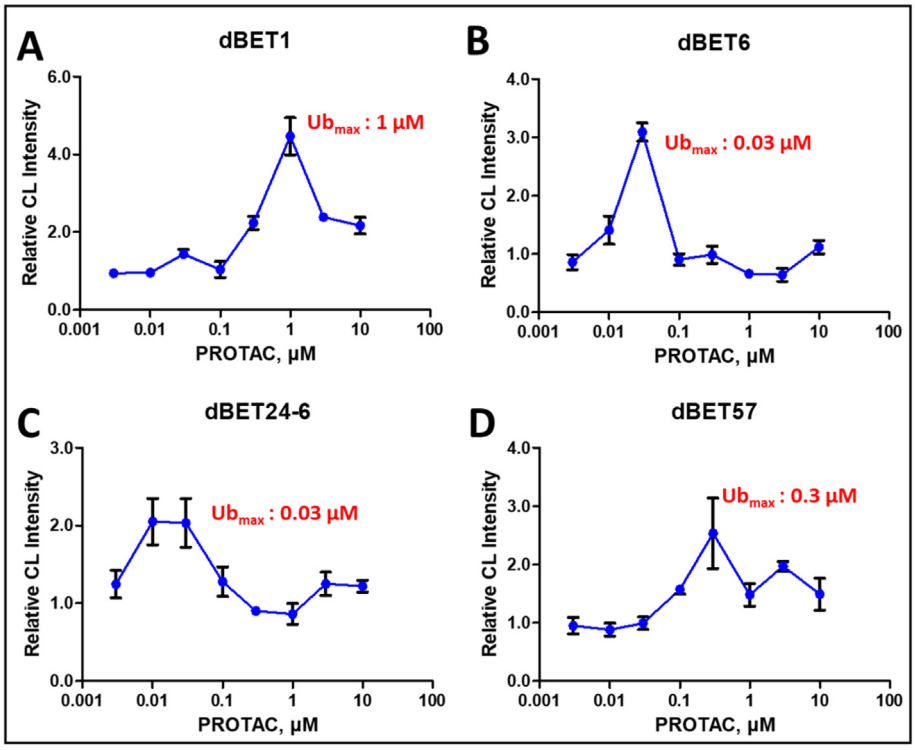

Fig. 2. TUBE based assay screening of PROTACs: Jurkat cell lysates were treated with BRD3-specific PROTACs A) dBET1, B) dBET6, C) BETd24-6, and D) dBET57. Polyubiquitination profiles and Ub_{max} of BRD3 for each PROTAC were represented as relative CL intensity. Relative CL intensities were calculated by dividing raw CL signals from a given PROTAC dose over DMSO treated samples. Error bars represent standard deviations, n = 3.

demonstrate the classic "hook effect" [26]. Degradation was measurable in cells treated with dBET1 at 3 μM (DC_{50}), with significant degradation up to 10 μM and no visible hook effect, consistent with Ub_{Max} of 1 μM observed in TUBE based PROTAC assay (Figs. 2A and 3). Cells treated with dBET6 degraded BRD3 with a DC_{50} of 0.03 μM and maximum degradation observed at 0.3 μM as well as a hook effect at 10 μM, correlating very well with our plate-based ubiquitination assay. BETd-24-6 showed enhanced degradation profiles with DC_{50} between 0.01 and 0.03 μM with no pronounced hook effect. This matched well with our plate-based assays where we observed Ub_{Max} sustained between 0.01 and 0.03 μM. Additionally, our plate-based assay demonstrated polyubiquitinated species reaching basal level at 0.1 μM (Fig. 2C), as well as western blotting represents complete loss of protein at 0.1 μM. Sensitivity limitations in western blotting may be attributed to the absence of a pronounced hook effect while TUBE based assays showed enhanced polyubiquitinated species post 1.0 μM (Fig. 2C) could be an indication for hook effect. We hypothesize that for potent PROTACs like BETd24-6 at higher doses, ternary complex formation may not be completely compromised and show sustained degradation. dBET57 treatment resulted in BRD3 degradation at 1.0 μM, with a DC_{50} of 1.0 μM and a D_{Max} at 3.0 μM. Pretreatment of the proteasome inhibitor MG132 with samples incubated with 0.03, 0.3, and 3 μM PROTAC rescued BRD3 in

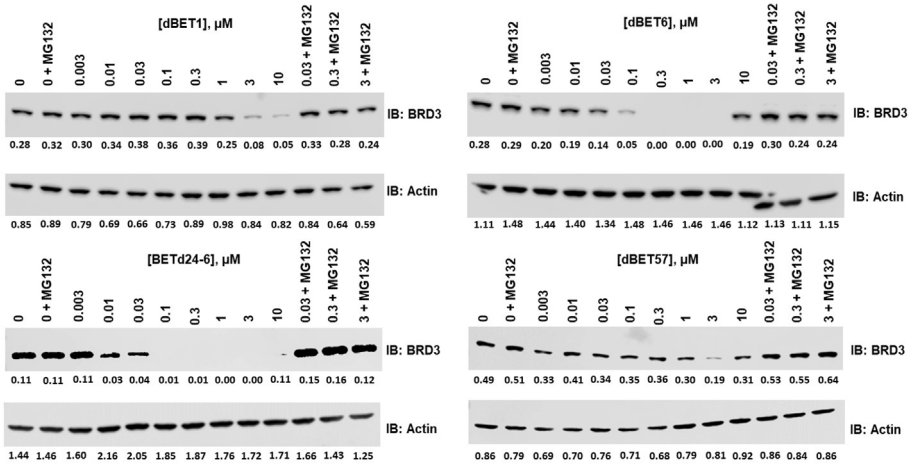

Fig. 3. PROTAC mediated degradation of bromodomain proteins analyzed by anti-BRD3 western blotting. Dose response of PROTACs dBET1, dBET6, Betd-24-6 and dBET57 at 45 min in Jurkat cells demonstrates degradation of BRD3, Acting as loading control.

western blotting, confirming PROTAC-mediated proteasomal degradation. A summary table showing comparison between Ub_{Max} and Ub_{Deg} from TUBE based assay and DC_{50} and D_{Max} from Western blot analysis is presented in SI, Table S2. Other than some variabilities owing to sensitivity challenges of western blots we noticed remarkable correlation. Comparative representation of TUBE based assay ubiquitination response and densitometric analysis of degradation western blotting are represented in Fig. S2. All raw images for the Western blot analysis (Fig. S3) along with DC_{50} and D_{Max} calculations are presented in SI, Table S3. *Note:* The spliced actin signal in the Fig. 3 dBET6, is related to gel breakage and subsequent challenge with transfer on to Western blot membrane.

2.1.1. Aurora a kinase degradation assays

Protein kinase networks are a primary component of cell signaling pathways and defects within these networks often lead to common human diseases such as neurodegeneration and cancer. Traditional kinase inhibitors are short-lived and require a significant intracellular drug concentration to work properly, leading to a variety of undesirable side-effects [27,28]. PROTAC degraders designed with highly promiscuous kinase inhibitor linked to a cereblon-binding ligand have shown to degrade many kinases [29]. We have explored Aurora kinase A (AURKA) a mitotic regulator, whose overexpression is prevalent in cancers [30,31], and one of the top kinases targeted by pan-kinase PROTACs, for its degradation kinetics using TUBE assays in K562 cells. Ternary complex formation, co-operative binding between E3 ligases and the target considered a rate limiting step and are often determined by biophysical studies [32]. While ternary complex formation and the associated K_D are helpful parameters to screen PROTACs for efficacy, cellular protein knockdown often depends on compound permeability, stability, target/E3 abundance, protein's intrinsic features, ubiquitination potential and re-synthesis rates [33,34]. Thus, understanding differences in PROTAC mediated ubiquitination rates among multiple PROTAC variants is crucial for rationally designing potent degraders. Time course experiments to monitor rates of ubiquitination in cells treated with PROTAC can represent stable complex formation between the E3 ligase and its target. Rapid PROTAC dependent degradation of a target can be readily attributed to slower dissociation rates and a longer half-life of the ternary intermediate species. In support of this statement, we observed robust and rapid intracellular ubiquitination of AURKA when treated with a promiscuous kinase PROTAC, TL12-186, previously used by Huang et al. [29].

Observing both trends in ubiquitination and the extent of ubiquitination (Ub_{Max}) is crucial when evaluating PROTACs. As shown in Fig. 3A, enhanced ubiquitination is observed over time, with Ub_{Max} occurring at 60 min and a ~3-fold increase over control (the zero-time point in samples treated with 100 nM of PROTAC, represented by the blue line). The red line in Fig. 4A and B represents the densitometric analysis of the Western blots, which monitors the degradation of AURKA. We observe that the levels of AURKA in samples treated with 100 nM of the promiscuous kinase PROTAC were reduced over time. The Western blot analyses are remarkably similar to the ubiquitination trends derived from our plate-based assays, establishing that 1) ubiquitination is the intermediate step prior to degradation and 2) ubiquitination kinetics control degradation. The Ub_{Max} and subsequent loss of the ubiquitinated signatures directly correlate with the precursor step of degradation in gel-based assays, indicating that our assay can be used to monitor PROTAC mediated ubiquitination and degradation simultaneously. Similarly, dose response studies employing concentrations ranging from 0.01 to 10 μM after 60-min of treatment have peak ubiquitination (Ub_{Max}) at 30–100 nM PROTAC concentrations and subsequent degradation at higher doses. Enhanced ubiquitination with increasing concentration suggests that concentrations beyond the DC_{50} of the PROTAC is no longer rate limiting. In many cases degradation levels off, as in dBET1 and dBET6 (Fig. 2A & B and Fig. 3), or in other cases an inhibitory hook effect is observed (e.g., AURKA, Fig. 4D). Either way, our plate-based assays have a strong correlation with Western blotting-based degradation assays (Fig. 4C&D). Raw images for the Western blot

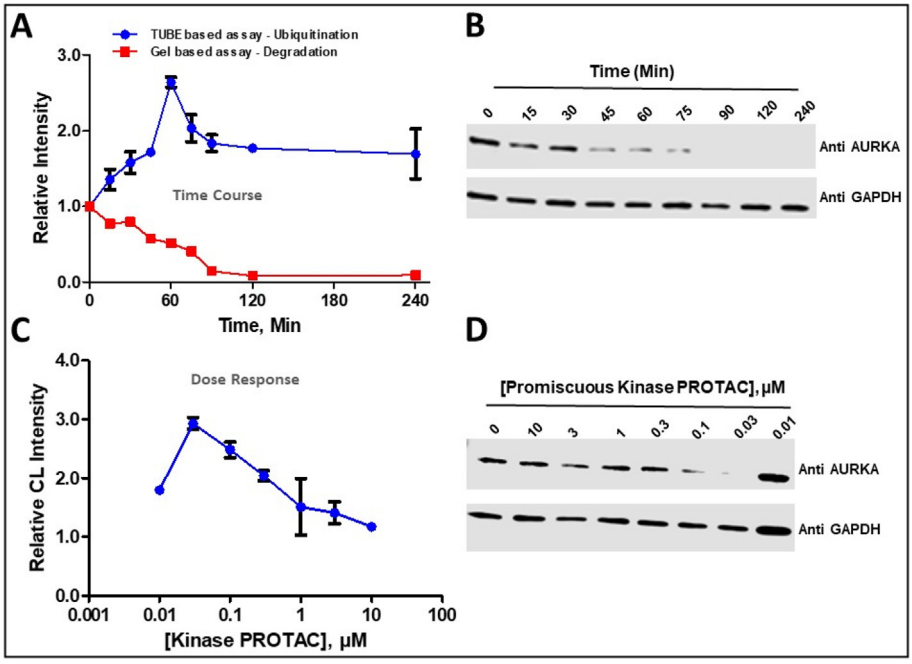

Fig. 4. PROTAC mediated ubiquitination and degradation of AURKA in K562 cells. (A) Time course study to evaluate intracellular ubiquitination and degradation. (B) Western blot analysis of time course study: degradation kinetics (C) A dose response study to evaluate DC_{50} of the promiscuous kinase PROTAC in K562 cells. (D) Western blot analysis of dose response study to monitor degradation, GAPDH as loading control. Error bars represent standard deviation, n = 3.

analysis for both time course and dose response are available in Fig. S4.

2.1.2. KRAS degradation assays

KRAS is the most frequently mutated oncogene in the Ras family of proteins known to mediate cell proliferation, differentiation, and survival [35]. The KRAS G12C mutation alone accounts for more than 44% of all KRAS mutations observed in non-small cell lung cancers (NSCLCs) and has been largely considered "undruggable" [36]. Sotorasib, recently approved by the FDA, selectively inhibits KRAS G12C to treat KRAS-driven cancers [37]. Several covalent inhibitors are available, including ARS-853, ARS-1620, AMG-510 (Amgen) and MRTX-849 (Mirati Therapeutics) [38] that have been explored for PROTAC applications to degrade endogenous KRAS G12C [39,40]. Bond et al. designed a VHL-based PROTAC, LC-2, with VHL E3 ligase, and Crews et al. designed a CRBN-based PROTAC based on the MRTX-849 ligand to successfully degrade KRAS G12C. While PROTACs designed by Zeng et al. [41] using ARS-1620 failed to induce endogenous KRAS G12C degradation despite successful ternary complex formation warrants need for efficient screening approaches to establish true PROTAC function i.e., ubiquitination and degradation is crucial for potent PROTAC discovery.

CRBN and VHL KRAS degraders were tested in heterozygous NCI–H358 cells. Both CRBN and VHL degraders were treated at 30 nM and 300 nM respectively, based on their predicted DC_{50} values in a time course experiment. As shown in Fig. 5A, in CRBN PROTAC treatments up to 18 h, we observed increased ubiquitinated KRAS (Ub_{Max}) levels at 2 h, with peak intensity ~3 fold compared to control (0 h). Loss of the ubiquitinated KRAS signal after 2 h, representing degradation post ubiquitination (shown in blue line). Comparative degradation responses obtained from Western blot densitometric analysis (Fig. 5A–C) shown in red line (Fig. 5A), indicating a very tight correlation between ubiquitination and degradation. VHL-based KRAS G12C PROTACs displayed enhanced ubiquitination, with peak intensities reaching ~7-fold and ~11-fold at 2 and 4 h respectively (Fig. 5B). Endogenous KRAS degradation rates strongly correlated with ubiquitination rates, with almost 50% degradation occurring between 2 and 4 h (Fig. 5B&C). Even though the KRAS ligand binds covalently, rapid degradation is not observed while BRD3 and AURKA PROTACs with reversible ligands showed peak ubiquitination and degradation within 1 h. Non-catalytic nature (slower dissociation rates) of the PROTACs designed from irreversible covalent KRAS G12C ligands might account for slower turnover of the protein. Not all E3 ligases even though they use same target ligand can degrade proteins efficiently. Our approach demonstrates that VHL degraders are relatively potent based on rate (2–4 h) and extent of ubiquitination (Ub_{Max} ~11 fold) when compared to CRBN degraders (Ub_{Max} at 3-fold enhancement at 2 h).

A dose response study to further investigate changes in potencies between CRBN and VHL G12C PROTAC was performed for 4 h with doses ranging from 10-0.01 μM. Cells were pretreated with MG132/DMSO for 1 h as needed to demonstrate proteasomal degradation. As expected, dose dependent ubiquitination and degradation of endogenous KRAS was observed (Fig. 6A&B). At higher doses, both CRBN and VHL degraders display covalent KRAS adducts, demonstrating successful PROTAC engagement (Fig. 6B). Fig. 6A, represents comparative analysis between CRBN and VHL degraders. Ubiquitination of KRAS with VHL degrader demonstrated Ub_{Max} at 0.1 μM, with peak intensity of 6-fold compared to ~2.5 fold at 0.03 μM with CRBN degrader. A typical dose response curve was observed with both the PROTACs validating ubiquitination event is precursor for degradation. For predicting ligase that is best suitable for enhanced degradability for KRAS, we have considered multiple factors 1. $Ub_{Max} \approx DC_{50}$ dose is a dose response

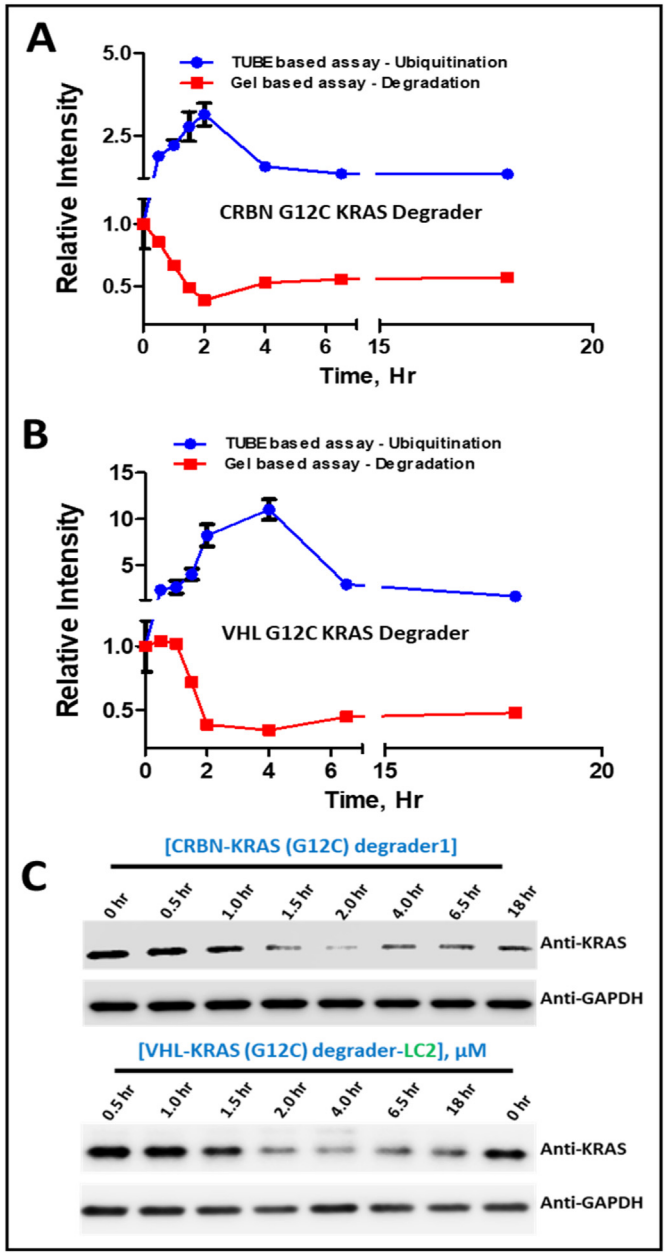

Fig. 5. Time course study to monitor ubiquitination and degradation kinetics of KRAS G12C degraders. (A) Ubiquitination and degradation profiles with a CRBN degrader at 30 nM with a comparison of Ub_{Max} to degradation rates using densitometric degradation analysis. (B) Ubiquitination and degradation profiles with VHL degrader at 300 nM with a comparison of Ub_{Max} to degradation rates using densitometric degradation analysis. (C) Western blot analysis with anti-KRAS to monitor degradation rates of KRAS with both CRBN and VHL PROTACs, GAPDH loading controls. Error bars represent standard deviation, n = 3.

analysis (CRBN – 0.03 μM and VHL – 0.1 μM) 2. Ubiquitination intensities in dose response studies (CRBN ~2.5 fold, VHL >2.5-fold at 0.03 μM and ~ 6-fold at 0.1 μM) 3. Time course studies showing dominant ubiquitination with VHL PROTACs. Studies with BRD3, AURKA and KRAS G12C have shown that a reliable fold increase in Ub_{Max} between ~ 2-fold and ~11-fold is suitable for PROTAC screening. Even tough extent of ubiquitination is crucial for degrading a target, to understand PROTAC efficiency we recommend considering a combination of DC_{50} dose and extent of ubiquitination. We demonstrate that VHL PROTAC based on Ub_{Max} fold

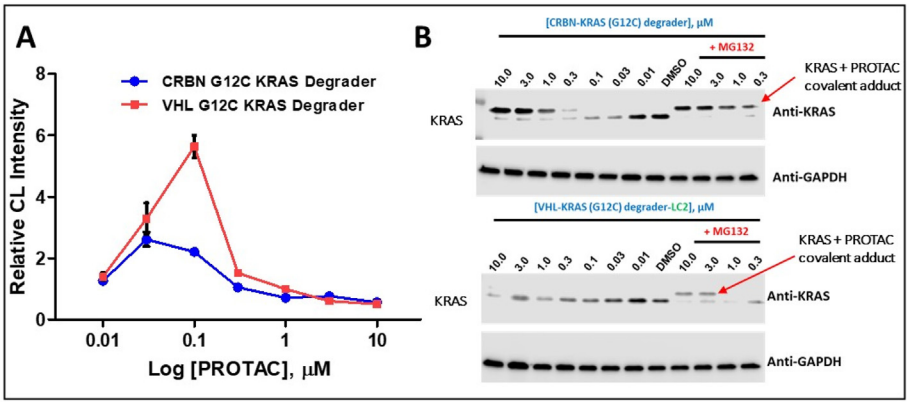

Fig. 6. Dose response study to monitor PROTAC-mediated ubiquitination and degradation (A) Plate-based assay: a comparative graph representing the ubiquitination response with CRBN and VHL PROTACs doses ranging from 0.01 to 10 μM, peak ubiquitination $Ub_{Max} \approx DC_{50}$. (B) Western blot analysis: degradation profiles of KRAS. GAPDH used as loading controls. Error bars represent standard deviation, n = 3.

increase and DC_{50} dose is more efficient compared to CRBN. Western blot analysis demonstrated similar dose-dependent degradation with DC_{50} values of 0.03 μM and 0.1 μM with CRBN and VHL degraders, respectively (Fig. 6B). Even though Western blot analysis suggests that the CRBN PROTAC has a lower DC_{50} compared to the VHL PROTAC sustained degradation of KRAS over a larger dose range consistent with enhanced levels of ubiquitination in the VHL PROTACs in TUBE assays. While MG132 treatment of cells containing CRBN PROTACs successfully rescued endogenous KRAS at 0.3 μM, the VHL PROTACs could not be fully rescued.

It is also worthwhile to note that Zeng et al. demonstrated lack of ubiquitination activity with ARS-1620 CRBN PROTAC is the reason for lack of degradation. We believe that the proposed ubiquitination screening platform can act as primary screen to monitor PROTAC variants for successful outcome. Our proposed platform is first of its kind to evaluate both ubiquitination and degradation simultaneously in a high throughput format for multiple ligands, different exit vectors, and different linkers to rapidly evaluate PROTACs function for endogenous targets. Proposed assay can demonstrate: 1. Functional cellular E3-target engagement; 2. formation of stable intermediate ubiquitinated species leading to degradation ($Ub_{Max} \approx DC_{50}$); 3. Dissociation rates and half-life of the intermediate ubiquitinated species (Kinetics-Time course studies: $Ub_{max} \approx$ maximum degradation). Ubiquitination studies can transform the PROTAC landscape by filling in some important gaps to accurately assess PROTAC and establish design criteria for medicinal chemists. While TUBE assays many other advantages like multiplexing, some challenges during data interpretation needs attention. 1. Hyper ubiquitination of target sometimes might cause masking of antibody binding epitope – a through antibody optimization to detect ubiquitinated targets will be beneficial in avoiding such scenarios. 2. Ambiguity between hook effect and degradation in some cases where PROTAC's hook effect, DC_{50} and D_{Max} doses are close. Thus, to eliminate false positives performing orthogonal assays like western blotting with top PROTAC candidates post TUBE assays will lay a strong basis for medicinal chemistry campaign in developing potent PROTACs.

3. Conclusions

The ubiquitin proteasome system is a highly regulated, complex series of protein-protein interactions that, even today, requires considerable research to be understood. Introduction of PROTACs created a need to build upon the collective knowledgebase of the UPS system. Nevertheless, establishing a link between ubiquitination and PROTAC-induced degradation is critical to make safe and efficacious degraders. Employing TUBEs as affinity reagents in a HTS format provides efficient and reliable PROTAC evaluation, improvement of SARs to design potent molecules and streamlining drug development. The proof-of-concept studies with three different targets and two ligases here demonstrate wide suitability. Our proposed TUBE-based approach can shed light on non-degradative PROTACs. We believe the proposed assays will be a valuable tool to research community for deeper investigations into the more complex mechanisms of the UPS relating to disease pathology.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Karteek Kadimisetty reports financial support was provided by LifeSensors Inc. Karteek Kadimisetty reports a relationship with LifeSensors Inc that includes: employment. LifeSensors Inc is not involved in study design, analysis, interpretation of the data and decision to submit.

The authors declare the following competing financial interest(s): The work is funded by LifeSensor Inc. All the authors in this study are employees of Lifesensors Inc. and LifeSensors Inc is the commercial owner of TUBE technology and their applications along with PA950: PROTAC assay plates.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.08.048.

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