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Letters

A Nimbolide-Based Kinase Degrader Preferentially Degrades Oncogenic BCR-ABL

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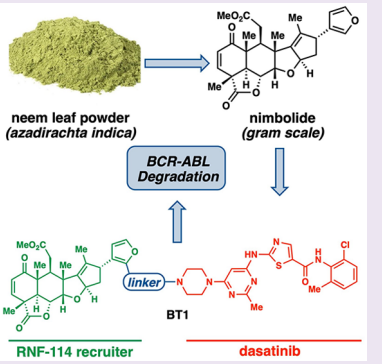
ABSTRACT: Targeted protein degradation (TPD) and proteolysis-targeting chimeras (PROTACs) have arisen as powerful therapeutic modalities for degrading specific proteins in a proteasome-dependent manner. However, a major limitation of TPD is the lack of E3 ligase recruiters. Recently, we discovered the natural product nimbolide as a covalent recruiter for the E3 ligase RNF114. Here, we show the broader utility of nimbolide as an E3 ligase recruiter for TPD applications. We demonstrate that a PROTAC linking nimbolide to the kinase and BCR-ABL fusion oncogene inhibitor dasatinib, BT1, selectively degrades BCR-ABL over c-ABL in leukemia cancer cells, compared to previously reported cereblon or VHL-recruiting BCR-ABL degraders that show opposite selectivity or, in some cases, inactivity. Thus, we further establish nimbolide as an additional general E3 ligase recruiter for PROTACs, and we demonstrate the importance of expanding upon the arsenal of E3 ligase recruiters, as such molecules confer differing selectivity for the degradation of neo-substrate proteins.



Targeted protein degradation (TPD) and proteolysis-targeting chimeras (PROTACs) are powerful therapeutic paradigms that employ heterobifunctional molecules to recruit an E3 ligase to a protein of interest for polyubiquitination and degradation by the proteasome.[1,2] While this technology is a very promising drug discovery paradigm for tackling so far intractable therapeutic targets, and small-molecule PROTACs have entered human clinical trials,[3] a major challenge in the application of this technology is the small number of known E3 ligase recruiters. While there are ∼600 different E3 ligases, many, for example, with different cellular localization, only a few E3 ligase recruiters have been identified and successfully employed, including small-molecule recruiters for cereblon (CRBN), VHL, MDM2, and cIAP;[4−9] among these, most reported degraders have used either CRBN or VHL ligands. In addition, recent reports also suggest resistance to degraders may occur through reprogramming of cellular machinery on the E3 ligase binding side, and there are certain proteins that have been resistant to degradation.[10,11] Therefore, the discovery of new E3 ligase recruiters is necessary to expand the scope of TPD applications.

Recently, chemoproteomic platforms have been used to discover additional recruiters that act through covalent targeting of cysteines on E3 ligases. These covalent recruiters include CCW16 that targets RNF4, SB002 that binds to DCAF16, and nimbolide that targets RNF114. To show proof-of-concept, these new recruiters were linked to JQ1 or to an SLF ligand to show proteasome-dependent degradation of BRD4 or FKBP12, respectively.[12−14] While these studies have effectively shown that E3 ligases can be targeted covalently for recruitment and degradation of their target proteins, both FKBP12 and BRD4 are among targets that are considered easily degradable, that is, many PROTACs have been developed that efficiently and robustly degrade these targets in a selective and proteasome-dependent manner.

In prior work employing nimbolide-JQ1 degraders, we observed selective degradation of BRD4 over related Bromodomain and extraterminal domain (BET) family members.[14] In this study, we further investigated the broader utility and selectivity of nimbolide-based PROTACs against protein targets that possess additional degradation selectivity challenges, namely, human kinases. Among these targets, selective targeting of the oncogenic fusion protein BCR-ABL, which is a driver of chronic myelogenous leukemia (CML), over c-ABL, an important nonreceptor tyrosine kinase involved in numerous cellular processes,[15] have proven to be challenging, with studied PROTACs exploiting CRBN or VHL recruitment. Pioneering studies by the Crews laboratory have shown that BCR-ABL PROTACs, consisting of CRBN or

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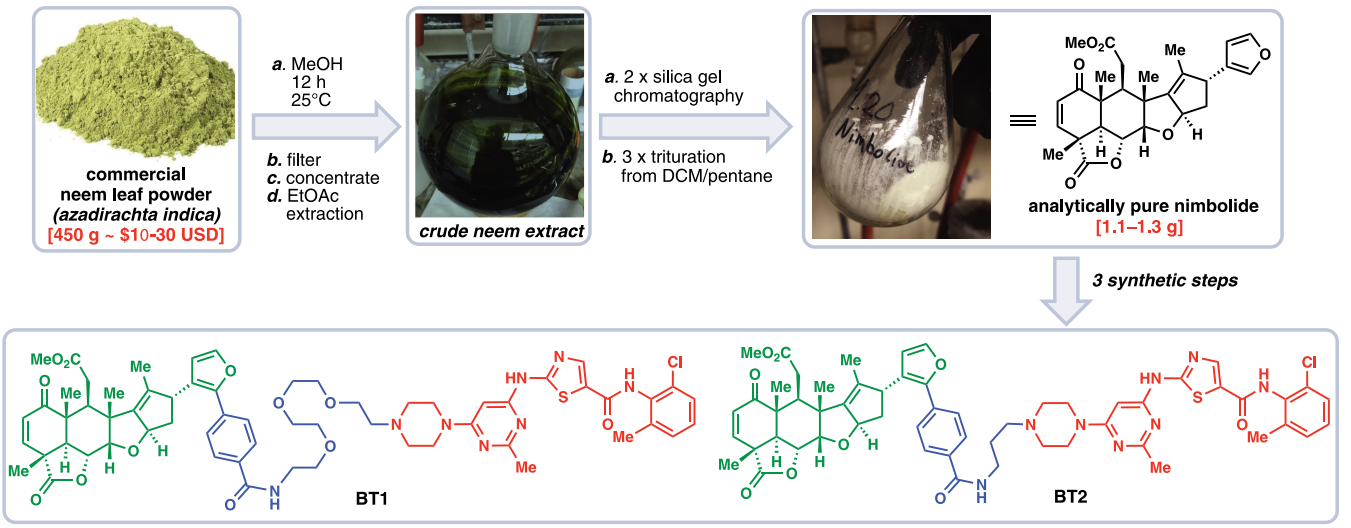

Figure 1. Extracting nimbolide from neem and nimbolide-based BCR-ABL degraders. Efficient method for extracting gram quantities of nimbolide from neem. This procedure is described in detail in the Supporting Information. Structures of the nimbolide-based BCR-ABL degraders BT1 and BT2 are shown.

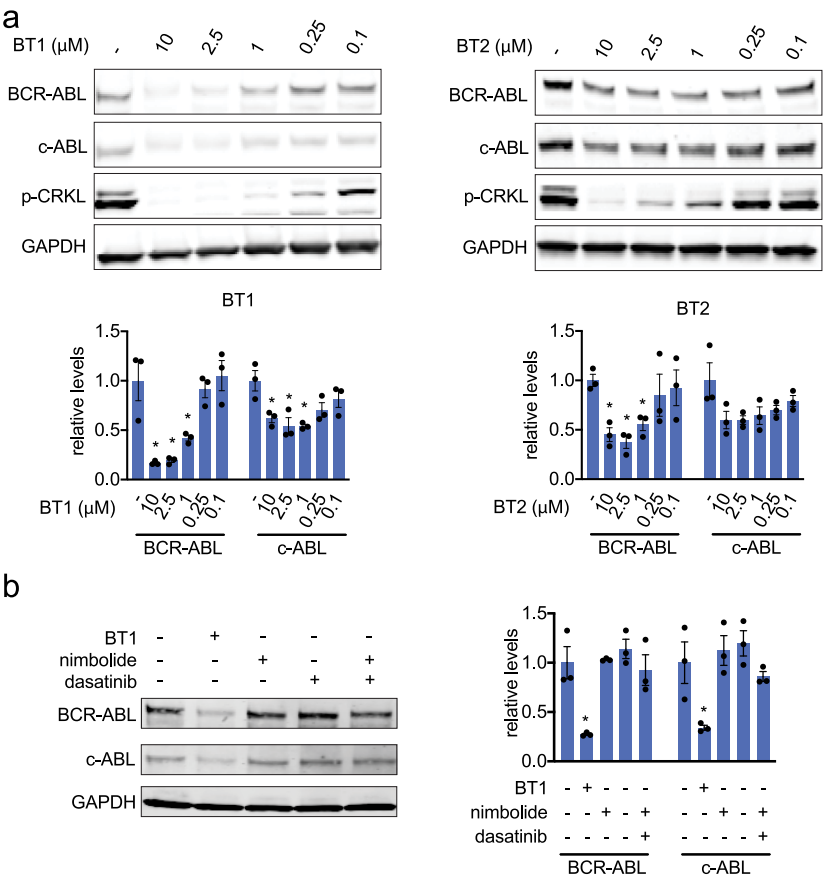

Figure 2. Effects of nimbolide-based BCR-ABL degraders on BCR-ABL and c-ABL: (a) BCR-ABL, c-ABL, phosphorylated CRKL, and loading control GAPDH levels with DMSO vehicle, BT1, and BT2 treatment in K562 cells for 24 h, assessed by Western blotting and quantified below in bar graphs by densitometry and normalization to GAPDH; and (b) BCR-ABL, c-ABL, and GAPDH loading control levels in K562 cells treated with BT1 (1 μM), nimbolide (1 μM), or dasatinib (1 μM) for 24 h, assessed by Western blotting and quantified by densitometry normalized to GAPDH. Blots are representative of $n = 3$ biological replicates per group. Quantified data show individual replicate values, average, and standardard error of measurement (sem). Statistical significance is expressed as $p < 0.05$ (denoted with an asterisk symbol, *), when compared to vehicle-treated control for each group.

VHL recruiters linked to BCR-ABL ATP binding pocket-targeting kinase inhibitors such as dasatinib and bosutinib or allosteric inhibitors such as GNF-2, showed preferential degradation of c-ABL over modest degradation of BCR-ABL.[16,17] Notably, this work has spurred the development of various degradation strategies targeting BCR-ABL.[18−22] In this

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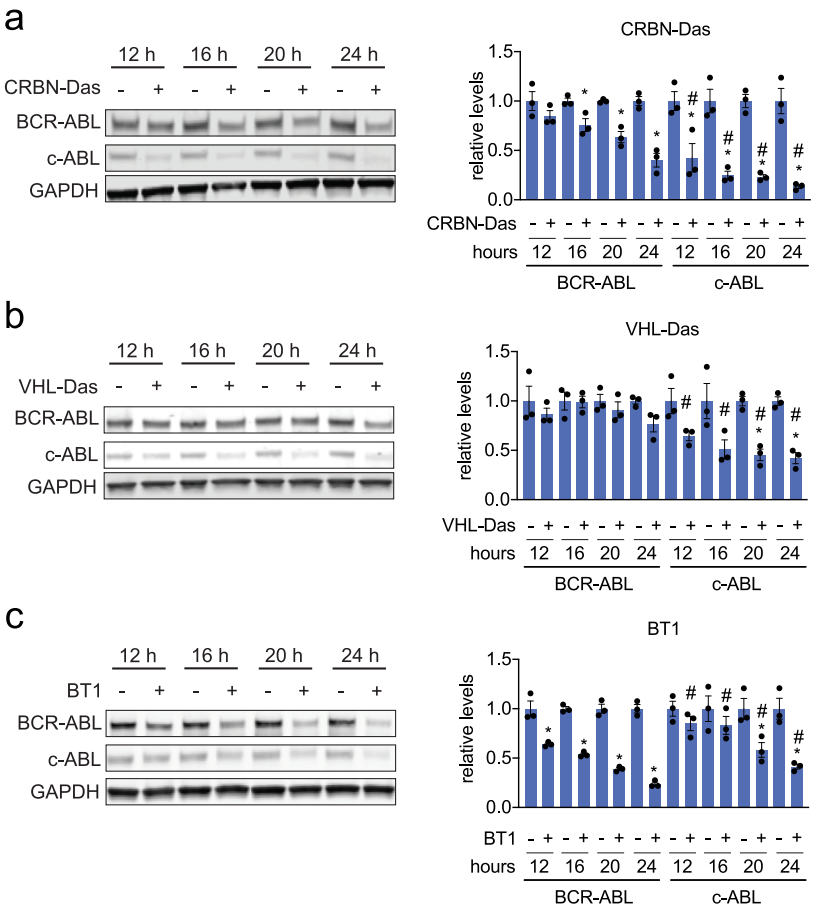

Figure 3. Rate of BCR-ABL versus c-ABL degradation with RNF114, CRBN, or VHL-based BCR-ABL degraders. (a, b, c) BCR-ABL, c-ABL, and loading control GAPDH levels with K562 cell treatment with DMSO vehicle or CRBN (pomalidomide)-, VHL (VHL ligand), or RNF114 (nimbolide)-based (BT1) dasatinib BCR-ABL degraders at 2.5 μM for 12, 16, 20, or 24 h, assessed by Western blotting and quantified by densitometry and normalized to GADPH loading control. Structures of all three degraders are shown in Figure S1 in the Supporting Information. Blots are representative of $n = 3$ biological replicates/group. Quantified data show individual replicate values, average, and sem. Statistical significance is expressed as $p < 0.05$ (denoted by an asterisk symbol (*), when compared to vehicle-treated control for each group, and a hashtag symbol (#), when compared to the corresponding BCR-ABL treatment comparisons with the individual degraders.

study, we aimed to determine whether our recently discovered covalent RNF114 recruiter nimbolide could be used to target and degrade kinases, and if so, whether differential selectivity in degrading BCR-ABL, compared to similar BCR-ABL PROTACs employing VHL or CRBN, could be attained.

The scope of targets amenable to nimbolide-based, RNF114-mediated protein degradation has, without question, been hampered by the extreme cost of nimbolide (2020 Sigma price = $77 000 USD/g), making small-scale, multistep synthetic manipulations of this acid-sensitive scaffold highly challenging. However, commercial neem leaf powder (*Azadirachta indica* extract) remains a popular and inexpensive health supplement containing varying amounts of nimbolide. Using a newly devised extraction protocol (see the Supporting Information), we were able to obtain gram quantities of analytically pure nimbolide from a single 1 lb can of Organic Veda neem leaf extract (2020 price ≈ $30 USD) without the need for HPLC purification (Figure 1). Importantly, this process opens the door to the synthetic and chemical biology communities to manipulate this complex triterpene scaffold for biological and especially TPD applications.

With access to significant quantities of nimbolide, we synthesized two nimbolide-based degraders linked to the tyrosine kinase inhibitor dasatinib employing both short alkyl (see BT2) and longer PEG-based (see BT1) linkers, in analogy to previous work (Figure 1; see the Supporting Information for synthesis details).[14] Treatment of K562 leukemia cells expressing the fusion oncogene BCR-ABL for 24 h with BT1 and BT2 led to the loss of both BCR-ABL and c-ABL, with a more pronounced effect observed with BT1 (Figure 2a). We were not able to determine whether higher concentrations of BT1 or BT2 led to impaired degradation because of "hook effects," previously observed with other PROTACs, including nimbolide-based degraders,[5,6,14] because of acute K562 cytotoxicity encountered at higher degrader concentrations. While our prior work on BRD4 degradation with Nimbolide/JQ-1-based PROTACS had found shorter linkers to be superior, we observed here that a longer linker between dasatinib and nimbolide showed better performance in degrading BCR-ABL and c-ABL, potentially because of improved positioning and formation of the ternary complex between RNF114 and the kinases, or because of increased accessibility of the degrader to the kinases in cells. Interestingly, both BT1 and BT2 showed more degradation of BCR-ABL than c-ABL, demonstrating opposite preference to Crews' findings (see Figure 2a). We show that, for phosphorylated CRKL, downstream of c-ABL, signaling was inhibited by both BT1 and BT2, indicating that BT1 and BT2

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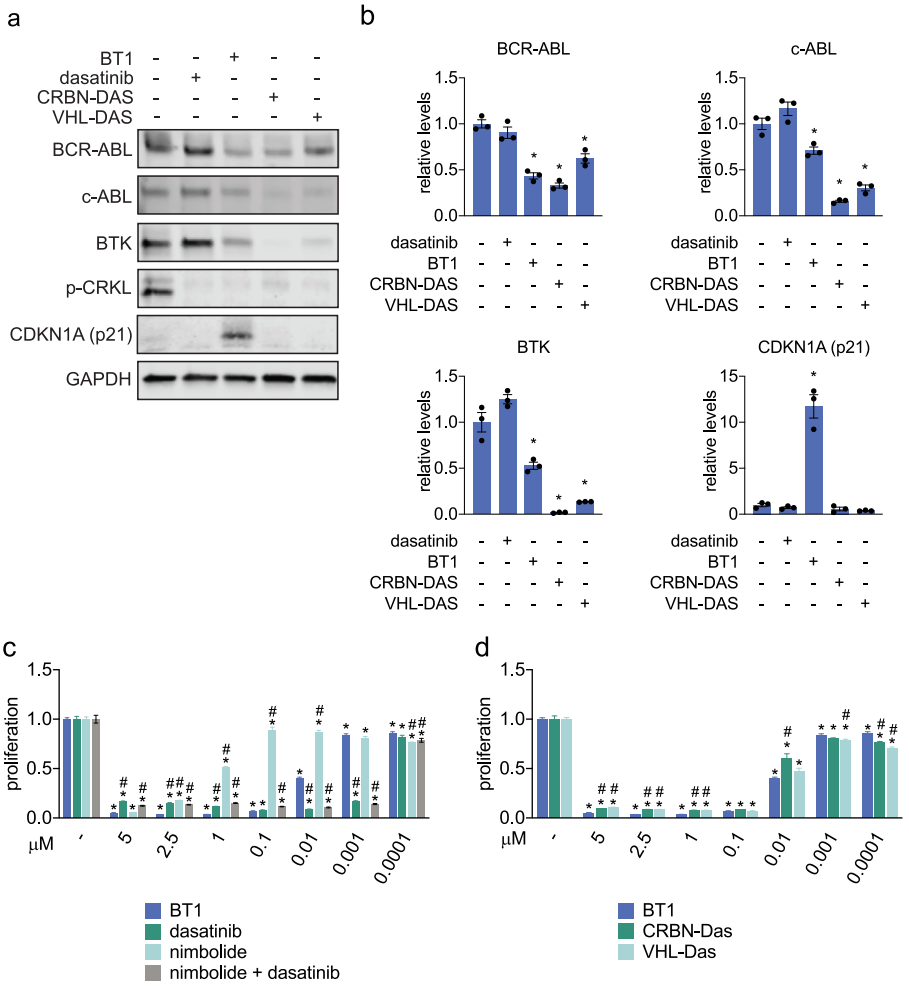

Figure 4. Comparing RNF114, CRBN, or VHL-recruiting BCR-ABL degraders. (a, b) BCR-ABL, c-ABL, BTK, phosphorylated CRKL, CDKN1A (p21), and loading control GAPDH levels in K562 cells treated with DMSO vehicle or dasatinib (2.5 μM), BT1 (2.5 μM), CRBN-DAS (2.5 μM), and VHL-DAS (2.5 μM) for 24 h, assessed by Western blotting and quantified in (b) by densitometry and normalized to GADPH loading control. (c, d) Cell proliferation of K562 cells treated with DMSO vehicle, BT1, dasatinib, nimbolide, nimbolide and dasatinib combined, CRBN-dasatinib, or VHL-dasatinib for 48 h. Blots are representative of $n = 3$ biological replicates/group. Quantified data show individual replicate values, average, and sem. Statistical significance is expressed as $p < 0.05$ (denoted by an asterisk symbol (*), when compared to vehicle-treated control for each group, or a hashtag symbol (#), when compared to the BT1 treatment group.

sufficiently engaged ABL in cells (Figure 2a). While performing rescue studies with proteasome inhibitors, NEDDylation inhibitors, or excess dasatinib or nimbolide was challenging, because of the acute toxicity of these compounds in this cell line, the degradation of BCR-ABL or c-ABL with BT1 treatment was not observed with nimbolide or dasatinib treatment, nor with cotreatment of both compounds (Figure 2b). We also attempted to knockdown or knockout RNF114 in this cell line, but RNF114 elimination in this line appeared to be lethal. Furthermore, attempted rescue experiments with BT1 in RNF114 knockout HAP1 cell lines, which we used previously in the context of BRD4 degradation,[9] were unsuccessful, since these cells did not express detectable levels of BTK or c-ABL, as judged by Western blotting. Nonetheless, our previous studies have convincingly demonstrated that nimbolide-JQ1 degraders reduced BRD4 levels in an RNF114- and proteasome-dependent manner.[14]

We next performed a more-detailed time-course analysis comparing our BT1 degrader with previously reported CRBN and VHL-recruiting dasatinib degraders of related linker length and composition (see Figures 3a–c, as well as Figure S1 in the Supporting Information). Consistent with previous reports, the CRBN-dasatinib PROTAC showed preferential and faster degradation of c-ABL, compared to BCR-ABL (Figure 3a). We also observed no significant degradation of BCR-ABL during the 24 h time course with >50% of degradation of c-ABL when employing the VHL-dasatinib degrader, consistent with Crews' findings (Figure 3b). In contrast, BT1 showed faster and preferential degradation of BCR-ABL over c-ABL at every time-point tested (Figure 3c). We also noted that BT1, CRBN-dasatinib, and VHL-dasatinib did not fully degrade BCR-ABL or c-ABL under the conditions tested. This incomplete degradation may be due to the faster rate of resynthesis of BCR-ABL and c-ABL in the cell, compared to the rate of degradation by the degraders. Alternatively, longer treatment times may result in more complete degradation. Another possibility may be that these degraders may preferentially degrade certain pools of BCR-ABL and c-ABL in the cell, such as nuclear versus cytosolic pools of BCR-ABL and c-ABL.[23]

We had previously reported that nimbolide disrupts endogenous RNF114 substrate recognition through targeting an N-terminal cysteine (Cys 8), leading to accumulation of

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endogenous RNF114 substrates such as the tumor suppressors CDKN1A (p21) and CDKN1C (p57), which, in turn, results in impaired breast cancer cell viability. Our previously described nimbolide-JQ1 BRD4 degrader showed selective degradation of BRD4, but also enhanced levels of p21 and p57 in breast cancer cells. Here, we demonstrate that BT1 treatment also led to greatly elevated levels of CDKN1A (p21) levels in K562 cells—an effect that was not observed with dasatinib, CRBN-dasatinib, or VHL-dasatinib treatments (see Figures 4a and 4b). These results indicate that a nimbolide-based BCR-ABL degrader may possess additional therapeutic properties beyond dasatinib alone. While dasatinib impaired K562 cell proliferation at much lower concentrations, compared to BT1, likely because of better cell permeability of dasatinib, compared to BT1, we observed significantly greater impairments in cell proliferation with higher concentrations of BT1 from 1 μM to 5 μM, compared to treatment with dasatinib alone, nimbolide alone, or even dasatinib and nimbolide together at time points of 24 and 48 h (see Figure 4c, as well as Figure S2 in the Supporting Information). We postulate that the observed heightened viability impairments observed with BT1, compared to dasatinib and nimbolide cotreatment, are due to the degradation rather than just inhibition of BCR-ABL (Figure 4c). We also observed significantly greater antiproliferative effects with BT1 from 1 μM to 5 μM, compared to CRBN-dasatinib and VHL-dasatinib PROTACs at time points of 24 and 48 h (Figure 4d; see Figure S2). This additional antiproliferative activity of BT1, compared to CRBN- or VHL-dasatinib PROTACs, may potentially be due to RNF114-mediated effects, such as p21 and p57 stabilization. We have previously shown that dual knockdown of p21 and p57 significantly attenuated the antiproliferative action of nimbolide in breast cancer cells.[14] Future studies genetically investigating the contributions of RNF114, p21, and p57 to the additional BT1-mediated antiproliferative effects would reveal greater insights into the mechanism of BT1 action. In addition, while CRBN-dasatinib potently ablates the known dasatinib target Bruton's tyrosine kinase (BTK), and VHL-dasatinib shows robust, but diminished degradation, substantially lower levels of degradation for these kinases are noted with BT1 treatment (see Figures 4a and 4b). We also attempted quantitative proteomic experiments on BT1 to look at degradation selectivity, but these studies proved to be challenging, because of the cytotoxicity induced by the time we observed robust BCR-ABL and c-ABL degradation necessary to detect total changes in ABL by proteomic methods (since we could not observe the BCR-ABL fusion tryptic peptide of the BCR-ABL protein). These findings further highlight the emerging complexities inherent to kinase degradation.[24−33]

Collectively, we demonstrate, for the first time, that covalent E3 ligase recruiters can be used to degrade kinase targets in TPD applications, and that a nimbolide-based BCR-ABL degrader shows unique degradation specificity profiles, compared with initially reported cereblon- or VHL-recruiting degraders. The propensity of RNF114-recruiting nimbolide-based degraders to degrade the oncogenic protein form more selectively is particularly notable. In addition, nimbolide-based degraders likely also possess additional anticancer effects through heightening the levels of tumor-suppressors such as p21; while complicating mechanistic studies on single targets, such polypharmacological intervention, represents a potentially attractive therapeutic strategy. Overall, our results underscore the importance of discovering more E3 ligase recruiters for better tuning the specificity and effects of future clinical candidates and drugs arising from TPD-based approaches. Finally, with ready access to supplies of nimbolide, a study of the scope of RNF114-mediated protein degradation can begin in earnest.

METHODS AND MATERIALS

Materials. Primary antibodies to ABL (Santa Cruz Biochemicals, No. 24-11), BTK (Cell Signaling Technologies, No. D3H5), GAPDH (Proteintech Group, Inc., No. 60004-1-Ig), phosphorylated CRKL Y207 (Cell Signaling Technologies, No. 3181), and p21 (Cell Signaling Technology, No. 12D1) were obtained from commercial sources and dilutions were prepared according to manufacturer recommendations. Antirabbit and antimouse seconadary antibodies were purchased from Licor (IRDye 800CW Goat anti-Rabbit IgG Secondary Antibody and IRDye 700CW Goat anti-Mouse IgG Secondary Antibody).

Isolation of Nimbolide and Synthesis of BT1 and BT2. Isolation of nimbolide and synthesis of BT1 and BT2 are described in the Supporting Information.

Cell Culture. K562 cells were obtained from the UC Berkeley Cell Culture Facility and were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% (v/v) fetal bovine serum (FBS), maintained at 37 °C with 5% CO_2.

Proliferation Assays. Cell proliferation assays were performed using WST8 reagent (APExBio, CCK-8), following the manufacturer's recommendations. K562 cells were seeded at a density of 100 000 cell/mL in a volume of 100 μL (10 000 cells per well).

Western Blotting. K562 cells were seeded for treatment at a density of 500 000 cells/mL, treated with compounds dissolved in DMSO, and harvested. Cell pellets were washed with 500 μL of phosphate buffered saline and lysed in 75−100 μL of radioimmunoprecipitation assay buffer (RIPA buffer) with a protease inhibitor cocktail (Thermo Fisher Scientific, Pierce Protease Inhibitor Mini Tablets, EDTA-free). Supernatant was transferred and total protein was normalized by Pierce BCA Protein Assay. Samples were denatured by addition of 4X Laemmli's Loading dye and 25−50 μg of protein was loaded onto 4%−20% TGX Precast gels (BioRad). After gel electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was then incubated for 1 h in 5% bovine serum albumin (BSA) in tris-buffered saline containing Tween 20 (TBST) before being incubated with the correct primary antibody overnight at 4 °C. The membranes were washed in TBST before a 1-h room-temperature incubation with secondary antibodies. After a final set of washes, blots were imaged on a LiCor CLX imager and band intensities were quantified using ImageJ software.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00348.

Supplemental extraction of nimbolide, synthetic methods, and supplemental figures (PDF)

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Notes

The authors declare the following competing financial interest(s): S.M.B., J.A.T., J.M.K., L.M., and M.S. are employees of Novartis Institutes for BioMedical Research. This study was funded by the Novartis Institutes for BioMedical Research and the Novartis-Berkeley Center for Proteomics and Chemistry Technologies. D.K.N. is a co-founder, shareholder, and adviser for Artris Therapeutics and Frontier Medicines.

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