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Bruton's Tyrosine Kinase Degradation as a Therapeutic Strategy for Cancer

Short title: **BTK Degradation as a Therapy for Cancer**

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KEY POINTS

- Small molecule-induced BTK degradation has superior antiproliferative effects than inhibition alone in cancer cells
- Lead degrader DD-03-171 reduces tumor burden and extends survival in lymphoma PDX models

ABSTRACT

The covalent Bruton's Tyrosine Kinase (BTK) inhibitor ibrutinib is highly efficacious against multiple B-cell malignancies. However, it is not selective for BTK and multiple mechanisms of resistance, including the C481S-BTK mutation, can compromise its efficacy. We hypothesized that small molecule-induced BTK degradation may overcome some of the limitations of traditional enzymatic inhibitors. Here, we demonstrate that BTK degradation results in potent suppression of signaling and proliferation in cancer cells, and that BTK degraders efficiently degrade C481S-BTK. Moreover, we discovered DD-03-171, an optimized lead compound that exhibits enhanced anti-proliferative effects on mantle cell lymphoma (MCL) cells *in vitro* by degrading BTK, IKFZ1 and IKFZ3 as well as efficacy against patient-derived xenografts *in vivo*. Thus, "triple degradation" may be an effective therapeutic approach for treating MCL and overcoming ibrutinib resistance, thereby addressing a major unmet need in the treatment of MCL and other B-cell lymphomas.

INTRODUCTION

Bruton's Tyrosine Kinase (BTK) is a TEC-family non-receptor tyrosine kinase that signals downstream of numerous cellular receptors, including the B-cell receptor (BCR), toll-like receptors (TLR), and Fc receptors (FcR)[1]. BTK plays a particularly important role in B-cell development and function, is critical for progression into cell cycle and proper B-cell activation[2-4], and loss-of-function mutations in BTK result in X-linked agammaglobulinemia due to a severe defect in B-cell development[5]. Importantly, BTK transduces constitutive signaling downstream of the BCR in many B-cell malignancies, so BTK has long been considered an attractive target for treating these diseases. Indeed, the clinically-approved covalent BTK inhibitor ibrutinib has been approved for use in patients with mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL), Waldenström's macroglobulinemia (WM), and marginal zone lymphoma (MZL)[6].

Despite ibrutinib's success in these indications, both intrinsic and acquired resistance have been observed in the clinic. For example, roughly one-third of patients with MCL fail to respond to ibrutinib (intrinsic resistance), which may be due in part to activation of non-classical NFκB signaling[7]. For those who do respond, acquired resistance quickly arises, often due to the C481S mutation of BTK, which prevents ibrutinib from forming a covalent bond with BTK and significantly reduces its potency, resulting in median progression-free survival of only approximately 14 months[8]. In fact, a "real-world" report of ibrutinib use in patients with MCL suggested that median time to progression or drug cessation due to toxicity is only 8 months[9]. Thus, the development of therapeutic strategies capable of preventing or overcoming BTK inhibitor resistance is an urgent unmet medical need for patients with MCL and other B-cell malignancies.

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Previously, we reported that HSP90 inhibition induced almost complete loss of BTK and other client proteins[10]. Correspondingly, HSP90 inhibition, through its effect on both BCR and non-classical NFκB signaling, reduced the viability of ibrutinib-sensitive and ibrutinib-resistant MCL cell lines, both *in vitro* and in patient-derived xenograft (PDX) models *in vivo*. However, to date there are no clinically approved HSP90 inhibitors, as most trials have been associated with limited efficacy and significant toxicity, presumably because HSP90 inhibition promotes degradation of different substrates in different tissues and rapidly induces stress responses that may mediate resistance[11].

Thus, we turned to a small molecule-mediated protein degradation platform that we and others have recently pioneered[12-15]. Small molecule degraders[16], also referred to as proteolysis targeting chimeras (PROTACs) or degronimids[17], contain an E3 ligase-targeting moiety connected via a linker to a ligand for the target of interest. Degraders bring an endogenous E3 ligase into close proximity with the target, leading to its ubiquitination and subsequent proteasomal degradation. Small molecule-induced degradation of proteins is an emerging pharmacological strategy that holds significant therapeutic promise[18], but not all ligandable targets are readily degradable. To determine which members of the kinome are amenable to this mode of pharmacological targeting, we previously generated a degrader that utilizes a promiscuous, multitargeted kinase inhibitor as its warhead[14]. While this compound efficiently bound to a large subset of the kinome, proteomic analysis revealed that not all kinases bound were effectively degraded. Notably, BTK scored as one of the most degraded kinases, indicating that it is a tractable target.

One of the most commonly employed E3 ligase ligands is thalidomide and its derivatives lenalidomide and pomalidomide, commonly referred to as IMiDs (immunomodulatory imide drugs). These agents are small molecule ligands of Cereblon (CRBN)[19], a substrate adaptor for the ubiquitously expressed cullin ring ligase 4 (CUL4)-RBX1-DDB1-CRBN ($CUL4^{CRBN}$) E3 ligase. Interestingly, thalidomide interacts with CRBN to form a novel surface, resulting in interactions with neo-substrates such as Ikaros (IKZF1) and Aiolos (IKZF3) and their ubiquitination and subsequent proteasomal degradation[20,21]. This activity alone has potent anti-tumor effects in some liquid malignancies, and lenalidomide (Revlimid®) is FDA approved for the treatment of MCL, multiple myeloma (MM), and myelodysplastic syndromes (MDS) with deletion of chromosome 5q. Lenalidomide is also undergoing late-stage clinical trials for a number of lymphomas, including MCL and the activated B-cell subtype of diffuse large B-cell lymphoma (ABC DLBCL).

Here, we investigated the utility of small molecule imide-based degraders of BTK. Specifically, we describe highly potent and selective BTK degraders with efficacy in cellular models of lymphoma and leukemia that can circumvent ibrutinib resistance. Moreover, we discovered that chemical structure modifications enabled us to tune IKZF1/3 degradation activity, allowing us to synthesize a degrader molecule that combined IKZF1/3 degradation with BTK degradation. Importantly, this “triple degrader” had enhanced potency against B-cell malignancies and significant efficacy in PDX models *in vivo*.

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METHODS

***In vitro CRBN-BTK dimerization*.** BODIPY-labeled CRBN in complex with Damage Specific DNA Binding Protein 1 (DDB1) harboring an internal deletion of the flexible BPB propeller (DDB1ΔB-CRBN)[22] and *in vitro* biotinylated BTK were treated with increasing concentrations of the indicated compounds in the presence of tracer amounts of Terbium-Streptavidin (Tb-SA). Compound-induced recruitment of BTK to CRBN was quantified following the 520/490 TR-FRET ratio[22] using a Pherastar plate reader (BMG) utilizing two synchronized PMTs to reduce background.

Cellular CRBN engagement. Cellular CRBN engagement was determined by interrogating the ability of BTK degraders or lenalidomide as control to outcompete binding of the BRD4 degrader dBET6 to CRBN. Cells stably expressing a $BRD4_{BD2}$-GFP fusion protein and mCherry from the same mRNA (separated by a P2A splice site)[22] were treated with dBET6 at 100 nM and increasing concentrations of BTK degraders or lenalidomide as control. The GFP/RFP signal ratio was quantified using an Acumen laser scanning cytometer (TTP labtech). Compounds capable of penetrating the cell membrane and binding to CRBN precluded dBET6 from inducing degradation of $BRD4_{BD2}$-GFP, resulting in a dose-dependent increase in GFP signal.

Cell lines. Mino, Granta-519, and Maver-1 cells were cultured in RPMI-1640 media supplemented with 10-20% FBS and 1% penicillin/streptomycin in a 37°C incubator with 5% CO_2. TMD8 and HBL1 cells were cultured in IMDM media supplemented with 10% FBS (Sigma) and 1% penicillin/streptomycin (Thermo Fisher Scientific) in a 37°C incubator with 5% CO_2.

C481S-BTK expressing cell models. Cells that overexpress wild type or BTK with Cys481Ser mutation (C481S-BTK) were prepared as described previously[23]. Briefly, TMD8 or HBL1 cells were transduced with lentiviral expression vector (pLVX-EF1α-IRES-Puro vector; Clontech Laboratories), with coding sequences for WT- or C481S-BTK. Following lentiviral transduction, stable cell lines were selected by 0.5~1.0 µg/ml puromycin. Equivalent expression for WT- and C481S-BTK in transduced cells was confirmed by immunoblots.

Western blots and antibodies. Cells were lysed in M-PER buffer (Thermo Scientific) containing protease/phosphatase inhibitor cocktail (Roche). Protein concentrations were measured using a BCA assay (Pierce). Equivalent amounts of protein for each sample were loaded on 4-12% Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes (BioRad), and immunoblotted with antibodies against BTK, HCK, phospho-Erk1/2 (Thr202/Tyr204), Erk1/2, phospho-S536-p65, p65, IKZF1, IKZF3, and Actin (Cell Signaling). IRDye®800-labeled goat anti-rabbit IgG and IRDye®680-labeled goat anti-mouse IgG (LI-COR) secondary antibodies were used and detected on an Odyssey CL_x system.

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Proliferation assays. Proliferation assays were performed by treating cells with compounds at the concentrations indicated for 72h. Anti-proliferative effects of compounds were assessed using CellTiter-Glo luminescent cell viability assays (Promega). ED_{50}s were calculated with Graphpad Prism nonlinear regression curve fit.

Synergy assays. The CellTiter-Glo assay was used to assess the dose-response of BTK inhibitor/degrader alone or in combination with other inhibitors. Compounds were dispensed using the JANUS Automated Workstation (PerkinElmer Inc.) into 384 well plates pre-seeded with cells by the MultiFlo™ FX dispenser (BioTek Instruments Inc.). Cells were incubated with compounds for 72h at 37ºC, and viability was assessed by luminescent measurements using the 2104 Envision® Multilabel Reader (PerkinElmer Inc.). Synergy was assessed by CalcuSyn 2.0 software (Biosoft, Cambridge UK) based on Chou TC[24].

LC-MS data analysis. Sample preparation and data acquisition for tandem mass tag mass spectrometry was carried out as previously described[25]. Proteome Discoverer 2.2 (Thermo Fisher) was used for RAW file processing and controlling peptide and protein level false discovery rates, assembling proteins from peptides, and protein quantification from peptides. MS/MS spectra were searched against a Uniprot human database (September 2016) with both the forward and reverse sequences. Database search criteria are as follows: tryptic with two missed cleavages, a precursor mass tolerance of 20 ppm, fragment ion mass tolerance of 0.6 Da, static alkylation of cysteine (57.02146 Da), static TMT labelling of lysine residues and N-termini of peptides (229.16293 Da), and variable oxidation of methionine (15.99491 Da). TMT reporter ion intensities were measured using a 0.003 Da window around the theoretical *m/z* for each reporter ion in the MS3 scan. Peptide spectral matches with poor quality MS3 spectra were excluded from quantitation (summed signal-to-noise across 10 channels < 200 and precursor isolation specificity < 0.5), and resulting data was filtered to only include proteins that had a minimum of 3 unique peptides identified. Reporter ion intensities were normalised and scaled using in-house scripts in the R framework[26]. Statistical analysis was carried out using the limma package within the R framework[27].

Generation of Animal Models. PDX models (Table S5) were engrafted in Nod.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/SzJ (NSG) mice purchased from Jackson Laboratories and handled according to the Dana-Farber Cancer Institute's Institutional Animal Care and Use Committee-approved protocol #13-034, as described[28]. Detailed description of models used and procedures can be found in the supplemental methods section and on www.PRoXe.org.

See supporting information for a description of protein docking, mass spectrometry, and chemical synthesis methods, and PDX model characterization.

Data sharing statement

Mass spectrometry raw data is available via the PRIDE archive under accession: PXD010568.

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RESULTS

Biochemical and mechanistic characterization of BTK degraders

To develop efficient degraders of BTK, we designed compounds based off of the previously reported selective BTK inhibitor CGI1746[29], and synthesized BTK degraders with polyethylene glycol (DD-03-007) or saturated hydrocarbon chain (DD-03-171) linkers conjugating the parent inhibitor to thalidomide (Figure 1A). As a negative control, we synthesized DD-03-033 (Figure 1A), an analog of DD-03-007 that lacks a carbonyl on the glutarimide ring of the thalidomide moiety, a modification that significantly reduces its affinity for CRBN[14]. We also generated DD-04-118 (Figure 1A), an analogue which differs from DD-03-171 by only a single atom change of the thalidomide aryl amine nitrogen to an oxygen, resulting in an aryl ether, a change that was predicted to compromise its ability to recruit IKZF1/3 to CRBN[22]. Crystal structures of inhibitor-bound BTK guided our selection of the piperazine as a suitable solvent-exposed linker attachment site. This selection was further validated by docking studies identifying the piperazine residue as an appropriate linker attachment site with the correct orientation of linker exit to bridge to an imide ligand bound to CRBN (Figure 1B).

To determine whether conjugation of E3 ligase binders to BTK inhibitors would impede the ability of these bivalent molecules to inhibit BTK, we tested these compounds in a commercial fluorescence resonance energy transfer (FRET)-based BTK kinase assay (Invitrogen Z'-Lyte; Table S1). Importantly, degraders had comparable activity to the parent inhibitor, validating our choice of linker attachment site. We also tested binding of DD-03-171 against a panel of 468 kinases (KINOMEscan) and found that, similar to CGI1746, DD-03-171 binds exclusively to BTK at a screening concentration of 1 μM (Figure 1C).

Next, we determined whether our CRBN-binding degraders could induce dimerization of CRBN and BTK. Using a time-resolved (TR)-FRET-based biochemical assay, we found that DD-03-007, DD-03-171, and DD-04-118 efficiently induced dimerization of BTK and CRBN, while neither lenalidomide nor the CRBN-nonbinding compound DD-03-033 could do so (Figure 1D). The broader peak of DD-03-171 vs. DD-03-007 may be because DD-03-171 exhibits greater cooperativity of BTK-degrader-CRBN complex formation.

Finally, we assessed cell permeability by interrogating each compound's ability to outcompete binding of the BRD4 degrader dBET6[30] to CRBN, and found that our BTK degraders had modest cell permeability compared to lenalidomide (Figure 1E).

Lead compounds potently and selectively degrade BTK in a proteasome- and CRBN-dependent manner

Next, we assessed the ability of these degrader compounds to destabilize BTK in Ramos B cells. Both DD-03-007 and DD-03-171 significantly reduced cellular BTK levels at concentrations as low as 100 nM within 4h of treatment while DD-03-033, a negative control that does not bind CRBN, did not (Figure 2A). This suggests that the cellular permeability of these compounds was sufficient to induce BTK degradation.

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Furthermore, both DD-03-007 and DD-03-171, but not DD-03-033, potently inhibited phosphorylation of Erk1/2, a downstream target of BTK, upon stimulation of the BCR (Figure 2B). To assess the durability of BTK degradation, we treated cells with DD-03-007 or DD-03-171 for 2h and collected cells at various time points after compound washout. Consistent with the extended half-life of BTK in primary B-cells[31], we found that BTK levels in degrader-treated cells remained below levels in vehicle-treated cells even 24h after washout (Figure 2C), illustrating that BTK degraders induce sustained depletion of BTK.

To verify the mechanisms of action of these BTK degraders, we co-treated cells with a proteasome inhibitor (bortezomib) or a NEDD8-activating enzyme E1 inhibitor (MLN-4924[32]) and found that both inhibitors prevented degradation of BTK (Figure 2D). Moreover, we performed competition experiments with either lenalidomide (for CRBN) or CGI1746 (for BTK) and found that excess quantities of either parent compounds could also prevent BTK degradation (Figure 2D). Thus, these BTK degraders act in a proteasome- and CRBN-dependent manner.

BTK degradation overcomes ibrutinib resistance and synergizes with HCK inhibition in Activated B-cell-like Diffuse Large B-cell Lymphoma (ABC DLBCL)

To explore the pharmacological consequences of BTK degradation in cellular models of cancer, we investigated the potential of our BTK degraders to overcome the increasingly urgent problem of acquired ibrutinib resistance in the clinic. While the most common ibrutinib-resistant BTK mutant is C481S, other mutants observed in patients include C481R, C481F, C481Y, T474I, and T474S[33]. New generation non-covalent BTK inhibitors have been developed that retain activity against the Cys481 and gatekeeper Thr474 point mutants[34]. Many of these noncovalent inhibitors have activity on WT and C481-mutant BTK with equal potency, and some even consistently inhibit all mutants. Thus, we hypothesized that degraders based on CGI1746 (an inhibitor with a similar structure and BTK binding mode as these new generation inhibitors) would be able to degrade clinically-relevant mutant forms of BTK.

To assess the ability of our degraders to overcome the C481S-BTK mutation, we treated TMD8 ABC DLBCL cells that expressed WT- or C481S-BTK with DD-03-007 and DD-03-171. Both degraders induced degradation of WT- and C481S-BTK, although DD-03-007 was slightly less potent (Figure 3A). We then compared the antiproliferative effects of CGI1746, ibrutinib, DD-03-007, and DD-03-171 on each cell line. While C481S-BTK expression reduced the potency of ibrutinib approximately 1,000-fold, it had minimal effects on the potency of DD-03-007 or DD-03-171 (Figure 3B); this was similarly true in HBL1 ABC DLBCL cells (Figure S1B). Based on previous reports, the potent effects of ibrutinib in BTK-wild-type TMD8 and HBL1 cells likely resulted from numerous off-target effects on other TEC and SRC family kinases[35-37], which are not recapitulated with the degraders. Taken together, these data illustrate that BTK degradation can overcome a clinically-relevant form of acquired ibrutinib resistance.

Consistent with the activity of ibrutinib on other TEC family kinases, its activity against HCK (in addition to BTK) is responsible for antiproliferative effects in L265P-MYD88-

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driven ABC DLBCL cells[37]. The parent inhibitor CGI1746 used for our degraders does not inhibit HCK[29], and our BTK degraders neither bind to nor degrade HCK (Figure 1C, 3A, 4B). Thus, we hypothesized that we could recapitulate the therapeutic benefits of ibrutinib's polypharmacology by combining BTK degradation with selective HCK inhibition. Consistent with this hypothesis, the HCK inhibitor A419259[37] exhibited strong synergy with both DD-03-007 and DD-03-171 in TMD8 cells at numerous doses (Figure 3C). Moreover, DD-03-171 maintained synergy with A419259 in TMD8 cells that express C481S-BTK (Figure S1A). Thus, combining BTK degradation with HCK inhibition (or other selective kinase inhibitors) may be a viable therapeutic strategy with reduced off-target effects compared to ibrutinib alone.

BTK degraders potently inhibit the proliferation of MCL cells

We next investigated the pharmacological effects of BTK degradation in MCL, a B-cell malignancy whose hallmark is the t(11:14) (q13:q32) chromosomal translocation between the cyclin D1 gene (*CCND1*) and the immunoglobulin heavy-chain locus (*IGH@*). Importantly, BTK is constitutively active in this disease, as a recent study found that despite similar BTK expression levels, levels of p-BTK (Y223), a surrogate marker of BTK activity, were higher in primary MCL cells than in resting B cells[38]. As such, ibrutinib and the more selective BTK inhibitor acalabrutinib[39] have shown beneficial effects in patients with MCL and have received FDA approval for this indication. Interestingly, an ongoing clinical trial of ibrutinib, lenalidomide, and the anti-CD20 antibody rituximab has reported promising initial responses[40]. As our degrader molecules contain moieties that have the potential to target both BTK and IKZF1/3, this presented us with the opportunity to determine the effects of co-degradation of BTK and IKZF1/3 in MCL cells.

First, we investigated whether DD-03-171 retained the activity of its imide moiety on IKZF1/3 and found that it potently degraded IKZF1/3 as well as BTK in Mino MCL cells (Figure 4A). In contrast, DD-04-118, which only differs from DD-03-171 by a single atom (Figure 1A), had minimal effects on IKZF1/3 levels (Figure 4A).

To more broadly assess the effects of degrader treatment on cellular protein levels, we performed multiplexed mass spectrometry-based proteomic analysis of Mino cells following 4h treatment (Figure 4B). Using abundance measurements from tandem mass tag (TMT) isobaric labels[41], whole proteome analysis resulted in the identification and quantification of >125,000 unique peptides corresponding to >9,000 proteins. Within the proteome, treatment with either DD-03-171 or DD-04-118 resulted in significant alterations in protein abundance of only two proteins each, and BTK was one of those proteins in both cases. Moreover, in DD-03-171-treated cells, IKZF1 and IKZF3 were degraded (~1.5-fold change for both proteins, which did not meet our minimum 2-fold change threshold to be pictured on the scatterplot); in contrast, in DD-04-118-treated cells, IKZF1 and IKZF3 levels did not change (Table S4).

Next, we found that DD-03-171 had potent antiproliferative effects on Mino cells (ED_{50} = 12 nM; Figure 4C). Consistent with our finding that ibrutinib synergizes with lenalidomide to inhibit the proliferation of Mino cells (Figure S2), DD-03-171 was more

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potent than the "single-degrader" compound DD-04-118 (ED_{50} = 69 nM), the parent compound CGI1746, and the MCL-indicated, clinically approved drugs lenalidomide and ibrutinib. DD-03-171 also outperformed CGI1746 and ibrutinib in inhibiting the proliferation of Maver-1 MCL cells, but none of the four compounds had significant effects in Granta-519 MCL cells (Figure S3).

As the NFκB pathway is critical in MCL cells[7], we examined the effects of BTK degradation on NFκB signaling in Mino cells. As expected, targeted degradation of BTK resulted in reduced levels of active (phosphorylated) p65 NFκB (Figure S4).

BTK degraders exhibit *in vivo* efficacy in MCL

Finally, we sought to investigate the effects of BTK degradation *in vivo*. To do so, we first compared the *in vivo* efficacy of DD-03-007 with DD-03-171 in terms of BTK degradation. Interestingly, while both compounds had similar pharmacokinetic profiles (Table S2–S3), only DD-03-171 induced significant degradation of BTK in nonmalignant mouse splenocytes (Figure S5).

After validating that DD-03-171 induced *in vivo* BTK degradation, we transitioned to *in vivo* PDX models of DLBCL (DFBL-18689) and MCL (DFBL-39435, DFBL-44685, DFBL-98848) (Table S5) engrafted into Nod.Scid.*IL2rγ*$^{-/-}$ (NSG) mice. Treatment with DD-03-171 resulted in *in vivo* degradation of BTK in all models, while degradation of IKZF1 varied (Figures S6, S7A, S8A, S9A). In DFBL-44685-engrafted mice, DD-03-171 treatment did not extend survival, which was not surprising as neither ibrutinib nor lenalidomide treatment affected survival (Figure S8B). In contrast, in DFBL-39435-engrafted mice, DD-03-171 treatment and the combination of ibrutinib and lenalidomide similarly reduced circulating tumor burden after 14-day treatment as compared to the control cohort (Figure S7B). Moreover, in DFBL-98848-engrafted mice, the reduction in circulating disease in DD-03-171-treated mice was at least comparable to mice treated with ibrutinib, lenalidomide, or ibrutinib and lenalidomide in combination (Figure S9B).

Encouraged by these results, we compared the efficacy of DD-03-171 to ibrutinib and lenalidomide in DFBL-96069-engrafted mice. This model was derived from a patient who previously failed multiple rounds of therapy, including chemotherapy and ibrutinib. Immunoblots from tumor samples of a cohort of mice treated for 3 days revealed extensive degradation of BTK in purified DFBL-96069 cells (Figure 4D). While DD-03-171 efficiently degraded IKZF1/3 *in vitro* (Figure 4A), the *in vivo* situation was more complex, as BTK inhibition alone induced upregulation of IKZF1/3. This upregulation was reduced in the DD-03-171-treated cohort, suggesting that IKZF1/3 degradation did occur but was not sufficient to overcome the compensatory upregulation of these two proteins in response to loss of BTK signaling (Figure 4D). Finally, we observed significantly reduced tumor burden in the peripheral blood and a significant extension of survival in mice treated with DD-03-171 compared to the other three cohorts (Figures 4E-F).

DISCUSSION

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Previously, by converting a multitargeted kinase inhibitor into a degrader, we profiled the degradable kinome and identified BTK as a tractable target[14]. Subsequently, several studies reported successful conversion of either a reversible ibrutinib scaffold[42,43] or a different noncovalent analog derived from a previously published covalent BTK inhibitor[44,45] into BTK degraders. Consistently, we found that CGI1746-based, CRBN-recruiting degraders with a variety of linker compositions potently induced cellular BTK degradation. Thus, BTK appears to be an easily degradable kinase.

Through rational linker engineering, we generated our lead compound DD-03-171, which combines degradation of BTK, IKZF1, and IKZF3, three validated targets in B-cell malignancies. The combination of ibrutinib, lenalidomide, and rituximab has reported promising initial results[40], raising the question of whether concurrent degradation of BTK and IKFZ1/3 is advantageous over treating patients with the combination of an IMiD and a BTK inhibitor. Theoretically, targeting multiple proteins with a single molecule avoids potential adverse drug-drug interactions and offers more predictable pharmacokinetics than a drug combination, as well as simpler dosing for patients. We envision that this intentional polypharmacological approach of degrading IKZF1/3 along with a distinct oncogenic driver may have broad utility as a strategy for targeting multiple cancer vulnerabilities, particularly in B-cell malignancies[46].

While several different BTK-binding ligands have been successfully converted into BTK degraders, it was unclear whether these compounds could efficiently induce BTK degradation *in vivo* and whether targeted BTK degradation would be a viable therapeutic modality. Here, we demonstrate that DD-03-171 has favorable pharmacokinetic and pharmacodynamic properties. To the best of our knowledge, this lead compound is the first targeted BTK degrader with demonstrated *in vivo* efficacy against B-cell malignancies. Nevertheless, there are several important factors to consider in weighing the relative risks and benefits of targeted BTK degradation. First, PROTACs may have suboptimal physicochemical properties for *in vivo* use. In a recent report of a BTK degrader, target degradation varied significantly between the spleen and lung despite similar distribution, potentially due to differences in the tissue-specific levels of CRBN.[44] Second, ibrutinib has multiple off-target effects (e.g. against HCK) that may contribute to its efficacy in specific contexts. Similarly, IMiDs are believed to have important effects independent of IKFZ1/3 degradation on the lymphoma microenvironment that contribute to their efficacy. It will be important to consider these off-target effects when designing studies using a triple-degrader like DD-03-171 in select populations. Third, there are multiple mechanisms through which a lymphoma cell may be resistant to BTK degraders, including reduced uptake, increased efflux, mutations of BTK that block binding, activating mutations in PLCγ2 or alternative NF-kB[7], or alterations that affect E3 ligase/proteasome function. Finally, BTK is essential for normal B-cell development and mice lacking IKZF1 have a complete block in B-cell differentiation at the pre-pro-B cell stage.[47] This suggests that a triple degrader could lead to profound B-cell lymphopenia akin to patients treated with anti-CD19 chimeric antigen receptor-T cells. These patients may require long-term supplementation with intravenous immune globulin and have increased risk for reactivation of Hepatitis B virus and other infections.

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In conclusion, we developed highly potent and selective small molecule degraders of BTK that exhibit a number of promising therapeutic characteristics, including: 1) durable degradation and potent inhibition of signaling and anti-proliferative effects; 2) an ability to overcome clinically-relevant forms of ibrutinib resistance; and 3) efficacy in PDX models *in vivo*. Thus, targeted BTK degradation is a novel and promising therapeutic approach for treating B-cell malignancies, potentially even those resistant to conventional therapies.

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Authorship
Contribution: D.M.W., N.S.G., E.S.F., and S.P.T. conceived and designed the experiments. D.D. designed and synthesized all compounds. E.S.W. performed all immunoblotting and proliferation experiments except those in TMD8 cells. S.M. and C.L. conducted the animal studies. R.P.N. performed docking analyses. K.A.D. performed the proteomics experiments. T.F. performed the biochemical assays. G.Y. prepared C481S-BTK expressing cell models, performed immunoblots, and antiproliferation experiments with TMD8 and HBL1 cells. Z.L. performed scale-up synthesis of DD-03-171 for animal studies. D.D. and E.S.W. wrote the manuscript.

Conflict-of-interest disclosure
N.S.G. is a founder and equity holder in Syros, Soltego, Petra and C4 Therapeutics. D.M.W. is a founder and equity holder in Travera and receives research support from AstraZeneca, Novartis, Aileron, Abbvie, Daiichi Sankyo, and Surface Oncology. E.S.F. is an equity holder and SAB member for C4 Therapeutics and receives research support from Novartis, and Astellas.

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FIGURE LEGENDS

Figure 1. Structure and biochemical characterization of BTK degraders. (A) Chemical structure and biochemical BTK IC_{50} of BTK degraders. (B) Docking of lenalidomide-bound CRBN (PDB 5FQD; shown as gray surface) with apo BTK (PDB 5P9J). BTK is overlaid with CGI1746-bound BTK (PDB 3OCS) shown in blue. (C) TREEspot visualization of the kinome selectivity profile of DD-03-171 (1 µM). (D) Compound-induced ternary complex formation of recombinant BTK and CRBN–DDB1 (Lanthascreen duplicate means +/- SEM). (E) Assessment of cellular CRBN engagement by displacement of dBET6 by a degrader. Cells stably expressing $BRD4_{BD2}$-GFP with mCherry reporter were treated with 100 nM dBET6 and titration of compound. Percentage of GFP/RFP ratio of RFP positive cells calculated from images using CellProfiler. Data is shown as means +/- s.d. of cell culture duplicates (n=2).

Figure 2. BTK degraders induce potent and sustained BTK degradation dependent on CRBN, neddylation, and the proteasome. (A) Immunoblots from Ramos B cells treated as indicated for 4h. (B) Immunoblots from Ramos B cells pre-treated with indicated compounds (1 µM) for 4h followed by stimulation with α-IgM (10 µg/ml) for 30 minutes. (C) Immunoblots from Ramos B cells treated for 2h with indicated compounds, followed by washout for indicated times. (D) Immunoblots from Ramos B cells pre-treated for 1h with bortezomib (500 nM), MLN9424 (1 µM), lenalidomide (10 µM), or CGI1746 (10 µM), and then treated with either DD-03-007 or DD-03-171 (100 nM) for 4h.

Figure 3. BTK degraders overcome ibrutinib resistance in activated B-cell diffuse large B-cell lymphoma (ABC DLBCL). (A) Immunoblots of TMD8 cells overexpressing WT- or C481S-BTK treated with the indicated compounds for 16h. (B) TMD8 cells overexpressing either WT- or C481S-BTK were treated for 3d with indicated compounds. Anti-proliferative effects of compounds were assessed using CellTiter-Glo assay kit (Promega), and ED_{50}s were determined using Graphpad Prism nonlinear regression curve fit. (C) TMD8 cells were treated with the indicated compound combinations for 3d. Plots show combination index (CI) scores. CI < 1 indicates synergy.

Figure 4. BTK degrader DD-03-171 shows on-target efficacy against MCL cells *in vitro* and *in vivo*. (A) Immunoblots from Mino cells treated as indicated for 18h; all bands for IKZF1 are specific. (B) Quantitative proteomics showing relative abundance of proteins in Mino cells treated with 200 nM of DD-03-171 or DD-04-118 for 4h. (C) Mino cells were treated with the indicated compounds and concentrations for 3d. Anti-proliferative effects of compounds were assessed using the CellTiter-Glo assay (Promega), and ED_{50}s were determined using Graphpad Prism nonlinear regression curve fit. (D) Immunoblot from mouse-depleted spleen samples of NSG mice engrafted with DFBL-96069 and treated for 3d with indicated compounds: vehicle (IP, BID), DD-03-171 (IP, QD, 50 mg/kg), lenalidomide (IP, QD, 50 mg/kg), ibrutinib (PO, QD, 30 mg/kg). All bands for IKZF1 are specific; each lane represents an individual tumor from different mice (3 mice per treatment cohort). (E) Tumor burden in mice treated for two weeks. 4-6h after the last dose, peripheral blood was quantified for disease burden via

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flow cytometry. * $p<0.05$ (F) Kaplan-Meier survival curve of mice treated as in (D) and (E).

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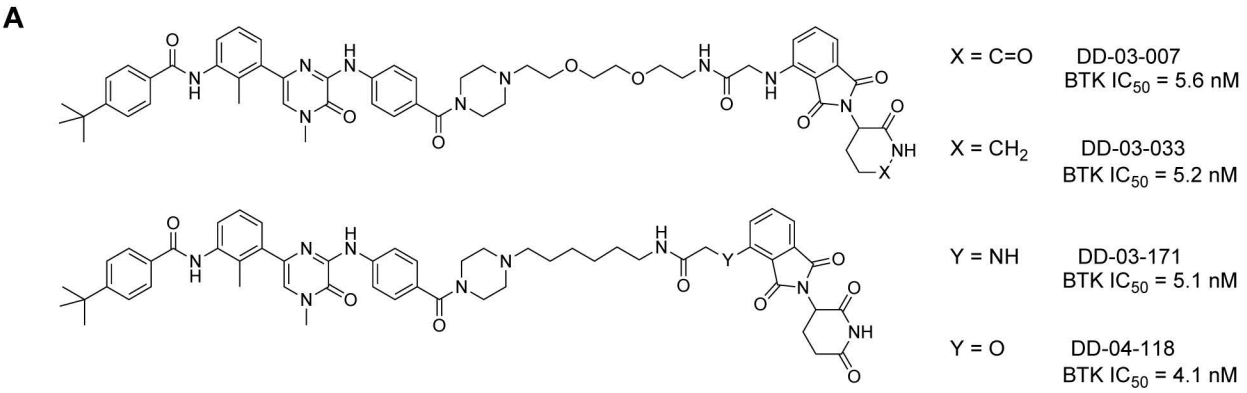

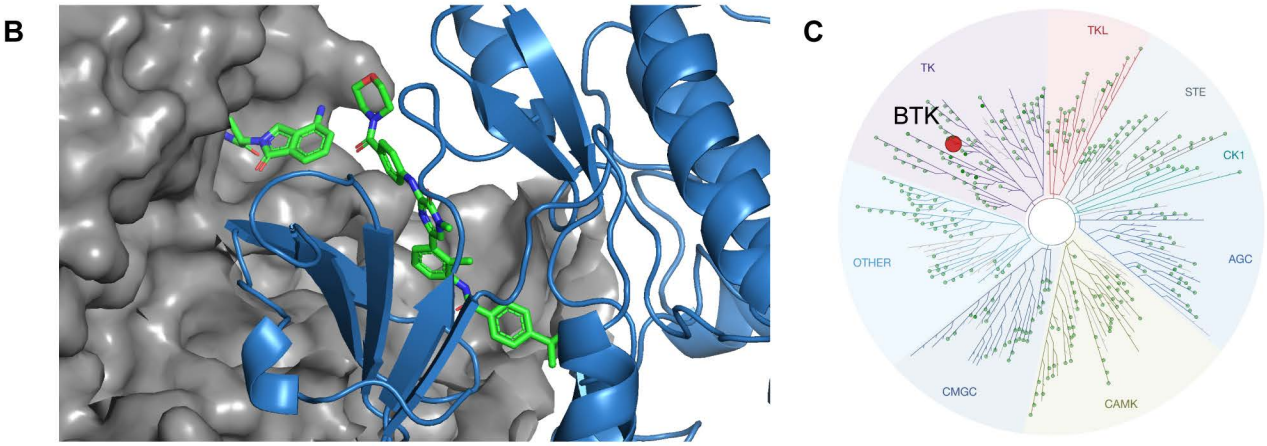

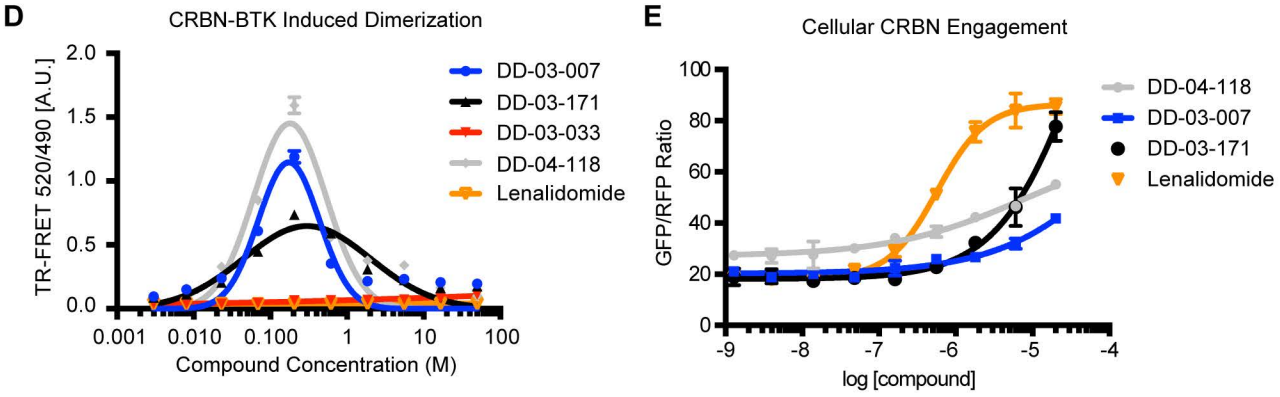

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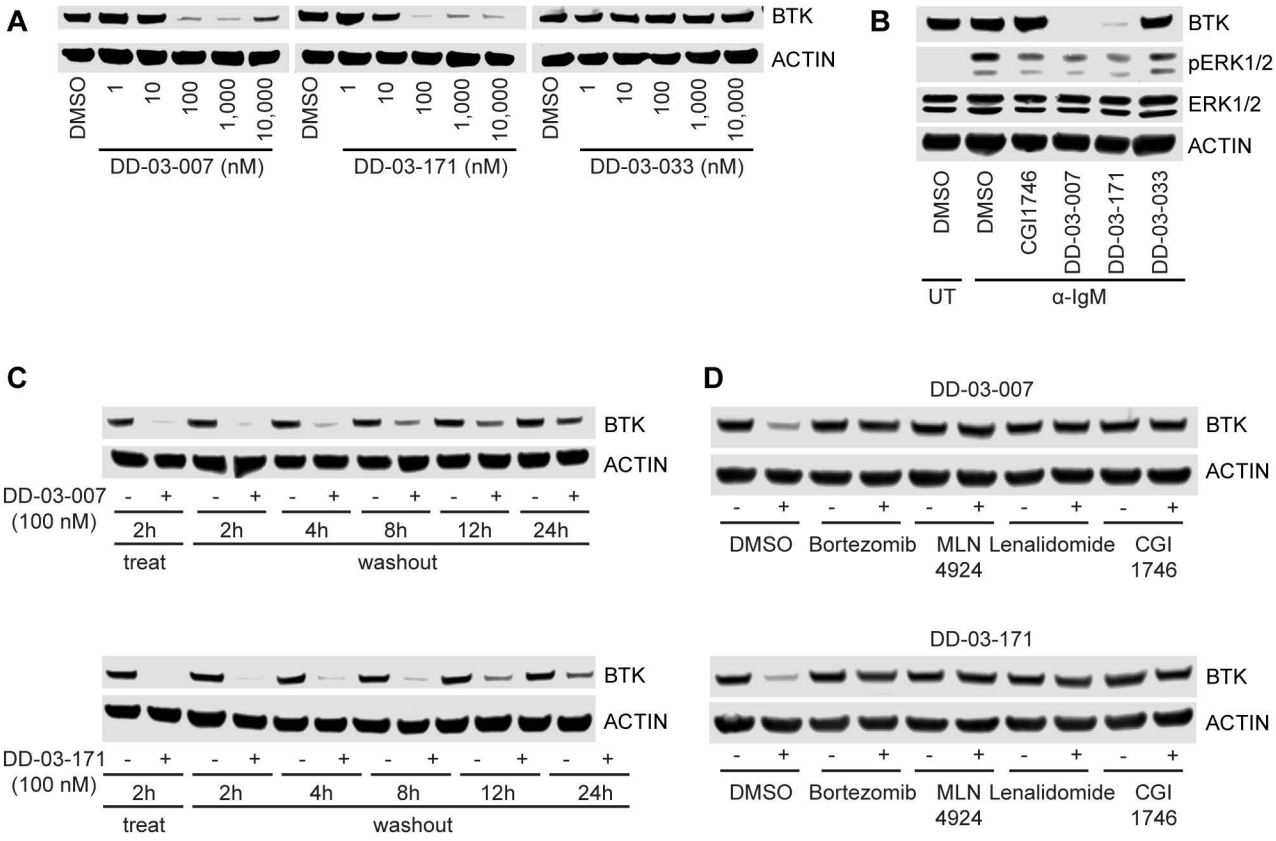

FIGURE 2

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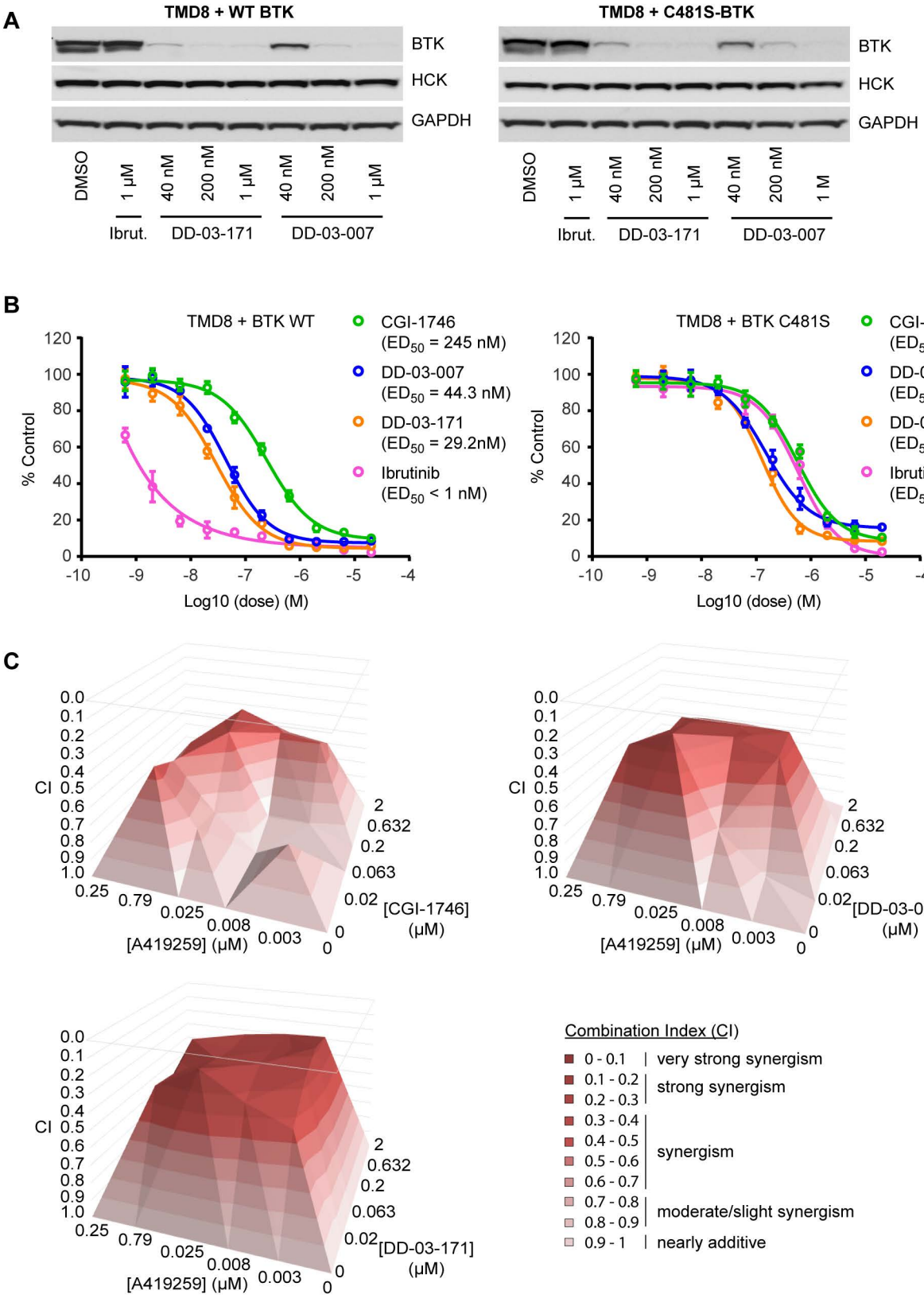

FIGURE 3

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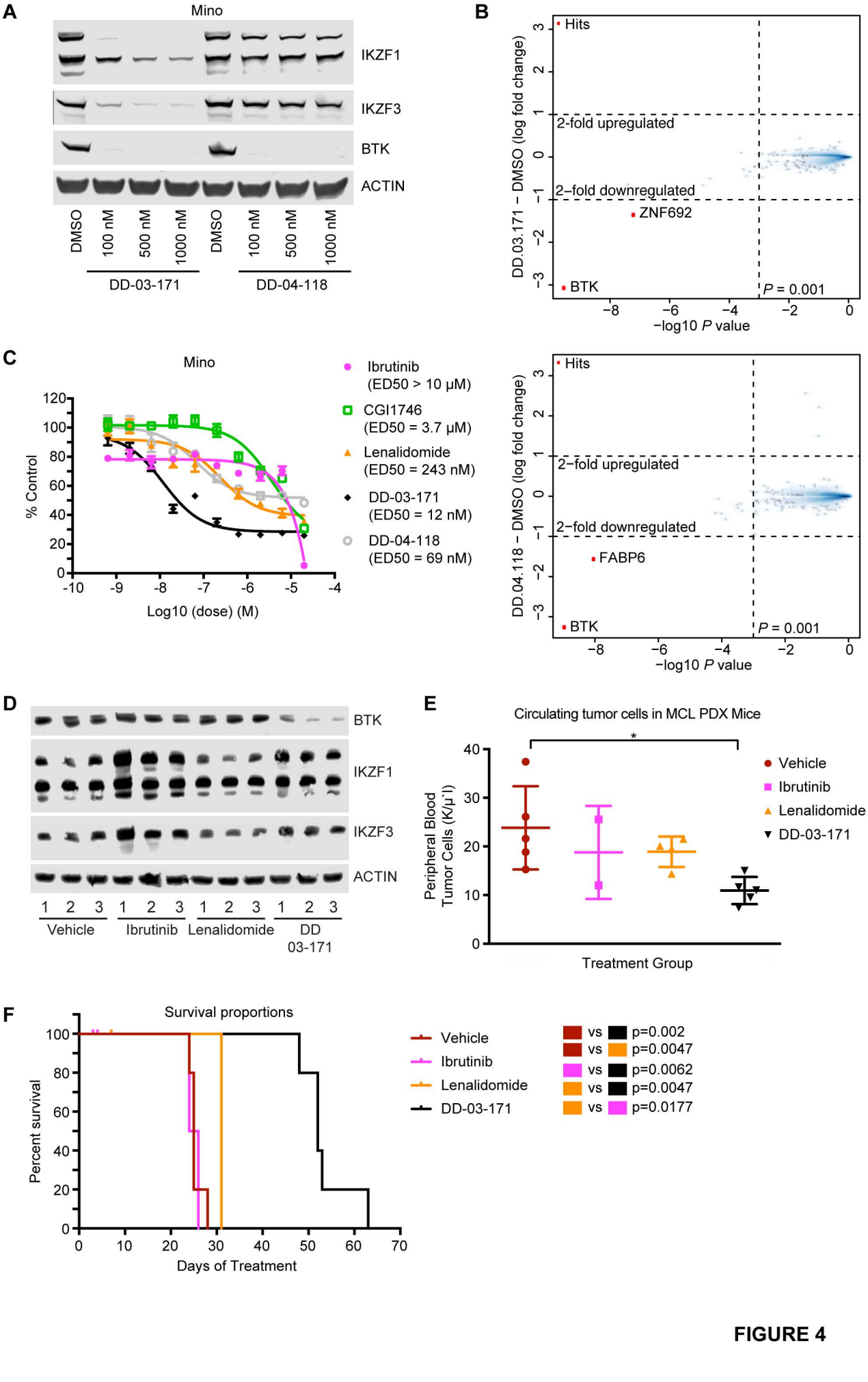

FIGURE 4

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Bruton's Tyrosine Kinase degradation as a therapeutic strategy for cancer

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