ABSTRACT

Thalidomide analogues exert their therapeutic effects by binding to the CRL4CRBN E3 ubiquitin ligase, promoting ubiquitination and subsequent proteasomal degradation of specific protein substrates. Drug-induced degradation of IKZF1 and IKZF3 in B-cell malignancies demonstrates the clinical utility of targeting disease-relevant transcription factors for degradation. Here, we found that avadomide (CC-122) induces CRBN-dependent ubiquitination and proteasomal degradation of ZMYM2 (ZNF198), a transcription factor involved in balanced chromosomal rearrangements with FGFR1 and FLT3 in aggressive forms of hematologic malignancies. The minimal drug-responsive element of ZMYM2 is a zinc-chelating MYM domain and is contained in the N-terminal portion of ZMYM2 that is universally included in the derived fusion proteins. We demonstrate that avadomide has the ability to induce proteasomal degradation of ZMYM2–FGFR1 and ZMYM2–FLT3 chimeric oncoproteins, both in vitro and in vivo. Our findings suggest that patients with hematologic malignancies harboring these ZMYM2 fusion proteins may benefit from avadomide treatment.

SIGNIFICANCE: We extend the potential clinical scope of thalidomide analogues by the identification of a novel avadomide-dependent CRL4CRBN substrate, ZMYM2. Avadomide induces ubiquitination and degradation of ZMYM2–FGFR1 and ZMYM2–FLT3, two chimeric oncoproteins involved in hematologic malignancies, providing a proof of concept for drug-induced degradation of transcription factor fusion proteins by thalidomide analogues.

INTRODUCTION

Thalidomide and its analogues, including lenalidomide and pomalidomide, have demonstrated extraordinary clinical utility mediated by drug-induced targeted protein degradation. Thalidomide analogues bind the Cullin-RING E3 ubiquitin ligase CUL4–RBX1–DDB1–CRBN (CRL4CRBN) complex, promoting ubiquitination and subsequent proteasomal degradation of non-target proteins, called neosubstrates (1–5). Thalidomide analogues bind a shallow hydrophobic pocket on the surface of CRBN to create a compound–protein interface for substrate recruitment to the CRL4CRBN complex, thereby acting as a “molecular glue” (6–8).

Thalidomide, lenalidomide, pomalidomide, and other thalidomide analogues induce degradation of IKZF1 and IKZF3, accounting for their therapeutic efficacy in multiple myeloma due to addiction of certain B-cell malignancies to these transcription factors (2, 4, 9). Lenalidomide-induced degradation of casein kinase 1 alpha (Ck1α) (10) explains the clinical efficacy of lenalidomide in myelodysplastic syndrome with deletion of chromosome 5q [del(5q); ref. 3]. Avadomide (CC-122) is a novel thalidomide analogue with potent antitumor and immunomodulatory activities, particularly in diffuse large B-cell lymphoma, through degradation of IKZF1/3 (10). Phase I trials revealed that avadomide monotherapy has acceptable safety and favorable pharmacokinetics (10–12).

Using quantitative mass spectrometry–based proteomic profiling, we found decreased ZMYM2 abundance following avadomide treatment. ZMYM2, formerly known as ZNF198, is a zinc finger protein that may function as part of a transcriptional corepressor complex (13). ZMYM2 has long been known for its implication in a chimeric fusion gene with fibroblast growth factor receptor-1 (FGFR1), generated by a t(8;13)(p11;q12) chromosomal translocation (14–19). ZMYM2 is the most frequent FGFR1 partner gene, accounting for 40% to 50% of cases of FGFR1-rearranged myeloid/lymphoid neoplasms (18, 20), a distinct entity in the 2016 revision of the WHO classification of myeloid neoplasms and acute leukemia (21). More recently, another fusion transcript involving ZMYM2, ZMYM2–FLT3, resulting from a cytogenetically cryptic inversion on chromosome 13, has been identified by RNA sequencing in patients with myeloproliferative neoplasms with eosinophilia (22) and Philadelphia chromosome–like acute lymphoblastic leukemia (23). In this study, we report for the first time that ZMYM2–FGFR1 and ZMYM2–FLT3 oncoproteins are avadomide-dependent CRBN neosubstrates, suggesting patients with hematologic malignancies harboring these ZMYM2 rearrangements may benefit from treatment with avadomide or novel thalidomide analogues that selectively degrade ZMYM2.
RESULTS

Avadomide Decreases ZMYM2 Protein Level

To identify novel targets of avadomide, we profiled changes in protein abundance in Hep3B cells, a hepatocellular carcinoma cell line, treated with varying concentrations of avadomide, pomalidomide, or vehicle control (Supplementary Fig. S1). Samples were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS-MS) employing isobaric chemical labeling and multiplexing using tandem mass tag (TMT) reagents for precise relative quantification and offline fractionation for deep-scale protein analysis. Of the ∼10,000 proteins quantified in Hep3B cells, 8 had decreased abundance following avadomide treatment relative to vehicle control, with a log2 fold change < -0.8 and a Padj value < 0.01 (Fig. 1A; Supplementary Fig. S2A-S2C; Supplementary Table S1). Among those significant hits were ZFP91, RAB28, and WIZ, previously identified as targets of thalidomide analogues (2, 24, 25), and two other proteins not previously published as drug-induced CRL4<sup>CRBN</sup> substrates, PDE6D and ZMYM2. Given the implication of ZMYM2 in two fusion oncoproteins involved in hematologic malignancies, we decided to prioritize this target for validation and follow-up studies. Pomalidomide, whose chemical structure is closely related to that of avadomide, had a less pronounced effect on ZMYM2 protein abundance (Fig. 1B).

To validate the proteomic findings, we performed immunoblot analysis in Hep3B cells and tested a series of thalidomide analogues. Avadomide was the only drug tested that robustly decreased ZMYM2 endogenous protein level. Pomalidomide had a relatively minor effect; lenalidomide, iberdomide (CC-220), and CC-885 had no detectable effect on ZMYM2 protein levels (Fig. 1C). Given the relevance of ZMYM2 in hematologic malignancies, we confirmed these findings in the acute myeloid leukemia cell line TF-1 ( Supplementary Fig. S2D). We next showed that avadomide decreased ZMYM2 protein abundance in a dose-dependent manner in multiple cellular contexts (Fig. 1D; Supplementary Fig. S2E and S2F). Furthermore, a time-course experiment revealed that the degradation kinetics of ZMYM2 were similar to ZFP91 in Hep3B cells (Fig. 1E), but relatively slower in TF-1 and JURKAT cells (Supplementary Fig. S2G and S2H). Avadomide decreased ZMYM2 protein levels without decreasing ZMYM2 mRNA levels (Fig. 1F; Supplementary Fig. S2I), consistent with a nontranscriptional mechanism of regulation.

Avadomide-Induced Ubiquitination of ZMYM2 Depends on CRBN

To investigate the mechanism of avadomide-mediated decrease in ZMYM2 protein level, we treated Hep3B cells with avadomide or the vehicle control for 12 hours in the presence or absence of small-molecule inhibitors of the ubiquitin-proteasome system. Cotreatment with avadomide and the E1 (UBA1) inhibitor MLN7243, which blocks all cellular ubiquitination by inhibiting the initial step of the ubiquitination cascade, abrogated ZMYM2 depletion. Likewise, addition of MLN4924, a NEDD8-activating enzyme inhibitor that interferes with the activity of Cullin-RING E3 ubiquitin ligases, or the proteasome inhibitor MG-132, prevented ZMYM2 from being degraded in the presence of avadomide (Fig. 1G).

We next sought to determine whether ubiquitination and degradation of ZMYM2 was dependent on CRBN, the molecular target of thalidomide analogues. We utilized CRISPR/Cas9 gene editing in Hep3B cells to generate biallelic inactivation of CRBN. In CRBN-knockout Hep3B cells, avadomide did not induce degradation of ZMYM2 (Fig. 1H). We next examined whether ZMYM2 binds CRBN and is ubiquitinated by CRL4<sup>CRBN</sup> E3 ubiquitin ligase. We observed coimmunoprecipitation of endogenous CRBN with V5-tagged ZMYM2 only in the presence of avadomide (Fig. 1I). Analysis of V5-tagged ZMYM2 in Hep3B cells with an antibody specific to K48-linked ubiquitin chains showed that avadomide treatment was associated with an increase in ZMYM2 carrying K48-linked polyubiquitin chains (Fig. 1J), the canonical signal for proteasomal degradation. Collectively, these results indicate that avadomide treatment causes CRBN-dependent ubiquitination and subsequent proteasomal degradation of ZMYM2.

ZMYM2 Degron Contains a C4 Motif

Thalidomide analogues in complex with CRL4<sup>CRBN</sup> recognize substrates through degrons, short stretches of primary sequence that are necessary and sufficient to mediate degradation (26). Many CRBN neosubstrates reported to date belong to the family of C2H2 zinc finger proteins, for which the drug-inducible degrons are zinc finger domains.

Figure 1. Avadomide promotes ZMYM2 degradation in a CRBN- and proteasome-dependent manner. A and B, Volcano plot illustrating changes in protein abundance assessed by proteomic analysis in Hep3B cells after 12 hours of treatment with 10 μmol/L avadomide (A) or 24 hours of treatment with 1 μmol/L pomalidomide (B). The plots represent statistical significance versus the fold change in protein abundance relative to the DMSO vehicle control. Results from two biological replicates are shown. Average log2 fold change in ZMYM2 protein abundance: -0.858, Padj value: 0.002 (A); -0.45, Padj value: 0.043 (B). C, Immunoblot analysis of ZMYM2, ZFP91, and GSP1 protein levels after 24 hour treatment with DMSO, 1 μmol/L lenalidomide (LEN), 1 μmol/L pomalidomide (POM), 1 μmol/L avadomide (AVA), 1 μmol/L iberdomide (IBER), or 0.005 μmol/L CC-985 in Hep3B cells. ZFP91 and GSP1 are shown as positive controls for degradation. D, Immunoblot analysis of ZMYM2 protein level in Hep3B cells treated with the indicated concentrations of avadomide for 24 hours. E, Time course of avadomide treatment in Hep3B cells for ZMYM2 protein level. F, Time course of avadomide treatment in Hep3B cells for ZMYM2 mRNA levels assessed by real-time qPCR. Data are mean ± SD; n = 3 technical replicates. G, Immunoblot analysis of ZMYM2 protein levels in Hep3B cells treated for 12 hours with DMSO or 20 μmol/L avadomide alone or in the presence of 0.5 μmol/L MLN7243, 5 μmol/L MLN4924, or 2 μmol/L MG-132. H, Immunoblot analysis of ZMYM2 protein level in wild-type or CRBN knockout Hep3B cells treated with avadomide for 24 hours. I, Immunoprecipitation of ZMYM2-V5 in Hep3B cells treated for 3 hours with DMSO or 10 μmol/L avadomide in the presence of 10 μmol/L MG-132. J, Ubiquitination analysis of ZMYM2-V5 in Hep3B cells treated with DMSO or the indicated concentrations of avadomide for 6 hours in the presence of 10 μmol/L MG-132. EV, empty vector; IP, immunoprecipitated. Results in C-J are representative of three independent experiments.
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(A) Avadomide

(B) Pomalidomide

(C) DMSO

(D) Avadomide

(E) Avadomide

(F) Avadomide

(G) Avadomide

(H) Avadomide

(I) Avadomide

(J) Avadomide
A conserved feature of this C2H2 zinc finger degrone is the canonical CXXCG motif (27), including a key glycine residue that engages the phthalimide moiety of thalidomide analogues (8, 27, 28). MYM-type zinc finger proteins, such as ZMYM2, are characterized by a conserved MYM domain with the consensus motif CX_2,CX_9–12,CX_5,CX_13–15,CX_9–25 FCX,CX_13–15 [F/Y] (15). By analogy to the C2H2 zinc finger proteins, we examined whether the CXXCG motif was also present in ZMYM2. This motif was found twice in the entire ZMYM2 protein, both located in the second MYM domain (MYM2; Fig. 2A). To test whether the MYM2 domain contained the degron, we employed a lentiviral degradation reporter vector that enables comparison of the fluorescence of enhanced GFP (EGFP)–tagged ZMYM2 construct to mCherry using flow cytometry (Fig. 2B; ref. 27). This assay revealed that the MYM2 domain (amino acids 425–502) was indeed sufficient to allow degradation in cells treated with avadomide. The MYM2 N-terminal portion (amino acids 425–466) similarly mediated degradation, whereas the C-terminal portion (amino acids 467–502) did not contribute. Further deletion mapping led to the identification of a ZMYM2 degron that was more sensitive to avadomide (amino acids 423–466), with a dose response almost overlapping with that of the IKZF1/3 degron (Fig. 2C). We next analyzed the effect of different thalidomide analogues on the degradation of the ZMYM2 423–466 degron in the reporter vector. Only avadomide and pomalidomide induced efficient degradation, while the other drugs had little or no effect, even at high concentrations (Fig. 2D; Supplementary Table S2).

To determine which specific amino acid residues are critical for ZMYM2 degradation, we performed an alanine scan, systematically mutating each amino acid within the 423–466 degron to alanine. Hep3B and TF-1 cells stably expressing the respective mutant degron in the degradation reporter vector were treated with avadomide or the vehicle control. The alanine scan highlighted seven residues critical for degradation in Hep3B and TF-1 cells, including four zinc-coordinating cysteine residues and three nonstructural amino acid residues, R424, I427, and the key glycine G429 in the conserved position against the bound avadomide molecule. The G429A mutant should not be tolerated in this binding mode, consistent with our degradation reporter and in vitro ubiquitination results. Of the other amino acid residues found to be critical for ZMYM2 degradation in our functional studies, R424 is proximal to avadomide in the ternary complex, and I427 makes direct contacts with the CRBN surface (Fig. 3). Therefore, both of these residues likely contribute to stabilization of the ternary complex needed for efficient ubiquitin transfer. Overall, this model confirms the interaction between the MYM2 domain of ZMYM2 and avadomide-bound CRBN. The model rationalizes the importance of the three functionally identified nonstructural amino acid residues in the ZMYM2 degron for CRBN binding and degradation.

**Avadomide Promotes ZMYM2–FGFR1 and ZMYM2–FLT3 Fusion Protein Degradation**

ZMYM2 is implicated in two chimeric fusion oncproteins involved in hematologic malignancies. In both fusion proteins, the N-terminal portion of ZMYM2, including the drug-responsive degron, is fused to the C-terminal portion of either FGFR1 or FLT3, including their respective tyrosine kinase domains (Fig. 4A; refs. 14, 15, 22, 23). No cancer cell lines harboring ZMYM2–FGFR1 or ZMYM2–FLT3 rearrangements are documented. To address whether the fusion proteins could be degraded in cells in a manner similar to the full-length ZMYM2 protein, we stably overexpressed the V5-tagged fusion proteins in the leukemia line TF-1 and treated these cells with avadomide for 24 hours. Immunoblot analysis using antibodies against the ZMYM2 N-terminus, FGFR1 or FLT3 C-terminus, and C-terminal V5 tag consistently showed that the ZMYM2–FGFR1 and ZMYM2–FLT3 proteins were both degraded upon avadomide treatment in a dose-dependent manner. The G429A point mutation within the ZMYM2 degron rendered both fusion proteins resistant to degradation, indicating that ZMYM2–FGFR1 and ZMYM2–FLT3 are degraded through the same mechanism as the full-length ZMYM2 protein (Fig. 4B and C).

Ba/F3 is a murine lymphoid leukemia cell line dependent on IL3, and its removal induces cell apoptosis. Like other tyrosine kinase oncogenes (29), ZMYM2–FGFR1 transforms Ba/F3 cells to IL3 independence (30, 31), thereby rendering Ba/F3 cells dependent on the fusion oncprotein for their...
Figure 2. Identification of the ZMYM2 degron and amino acids critical for drug-induced degradation. A, Sequence alignment of ZMYM2 MYM2 domain and the different peptides tested for degradation using the reporter assay. The cysteine residues that coordinate the zinc ions are highlighted in gray. The glycine residues following a CXXC motif are highlighted in yellow. B, Schematic of the protein degradation reporter vector used to identify the ZMYM2 degron. IRES, internal ribosome entry site. C, Hep3B cells stably expressing the ZMYM2 constructs in the degradation reporter were treated for 20 hours with DMSO or avadomide and analyzed by flow cytometry to quantify the DMSO-normalized ratio of EGFP/mCherry fluorescence. The dose–response curve for IKZF1/3 zinc finger 2 (ZF2) domain is represented as a reference (mean ± SD, n = 4 biological replicates). AA, amino acid. D, Effect of different thalidomide analogues on the degradation of the ZMYM2 423–466 wild-type degron in the reporter vector in Hep3B cells (mean ± SD, n = 4 biological replicates). IC50, inhibitory concentration 50; n.r., not reached. E, Alanine scan of the ZMYM2 423–466 degron using the reporter assay in Hep3B and TF-1 cells treated with 1 μmol/L avadomide for 20 hours. Amino acids that reduced degradation above 0.4 when mutated into an alanine are in red font (mean ± SD, n = 3 biological replicates). F, MBP-tagged ZMYM2 amino acids 423–466 wild-type or G429A were tested for in vitro ubiquitination by CRBN–CRL4 in the absence or presence of 10 μmol/L pomalidomide or avadomide. Ubiquitination reactions were separated by SDS-PAGE, followed by anti-MBP. G, Immunoblot validation of the ZMYM2 degron in Hep3B cells transfected with the empty vector, ZMYM2 wild-type, ZMYM2 with deletion of the MYM2 domain (del 425–502), or ZMYM2-binding mutant G429A and treated with DMSO or 10 μmol/L avadomide for 24 hours. Results in C–G are representative of three independent experiments.
survival and proliferation. We transformed Ba/F3 cells with ZMYM2–FGFR1 bearing the wild-type degron (ZMYM2WT–FGFR1) or its G429A mutant counterpart (ZMYM2G429A–FGFR1; Fig. 5A and B). Because mouse cells are intrinsically resistant to thalidomide and its analogues, transformed Ba/F3 cells were subsequently transduced to express either human CRBN or the humanized mouse CrbnI391V mutant, which are both capable of mediating drug-induced degradation of Izkf1/3, Ck1α, and Zip91 in murine cells (3, 32). Expression of human CRBN allowed robust degradation of ZMYM2WT–FGFR1 upon avadomide treatment. However, expression of CrbnI391V was not sufficient to promote degradation of human ZMYM2WT–FGFR1 (Fig. 5C), consistent with the lack of cytotoxic effects of avadomide in these cells (Supplementary Fig. S4).

Ba/F3 transformation by ZMYM2–FGFR1 is mediated, at least in part, by activation or subversion of signaling pathways employed by the Jak–Stat pathway downstream of activated receptor tyrosine kinase signaling (30). Immunoblot analysis showed that Stat1 and, to a lesser extent, Stat5 were phosphorylated in ZMYM2–FGFR1-transformed Ba/F3 cells. In Ba/F3 cells transformed by ZMYM2WT–FGFR1 and expressing human CRBN, avadomide treatment led to ZMYM2WT–FGFR1 degradation and a concomitant decrease in phospho-Stat1 and Stat5 levels (Fig. 5D). In Ba/F3 cells transformed with the drug-resistant ZMYM2G429A–FGFR1 fusion, the effects of avadomide on phospho-Stat1 and Stat5 were absent or decreased (Fig. 5E). In Ba/F3 cells transformed by ZMYM2WT–FGFR1 and expressing human CRBN, avadomide reduced cell viability and proliferation in a dose-dependent manner. This effect was completely rescued in Ba/F3 cells transformed by the ZMYM2G429A–FGFR1 mutant, confirming that the cytotoxic effect of avadomide was due to ZMYM2WT–FGFR1 depletion. Ba/F3 cells transformed by either ZMYM2WT–FGFR1 or ZMYM2G429A–FGFR1 were all sensitive to ponatinib, a multityrosine kinase inhibitor with activity against FGFR1 (33), regardless of CRBN status (Fig. 5F).

In Vivo Validation of ZMYM2–FGFR1 Degradation by Avadomide

To address whether avadomide could inhibit the proliferation of ZMYM2–FGFR1-dependent leukemia cells in vivo, we performed a syngeneic transplant experiment in BALB/c mouse recipients (34, 35), the mouse strain from which Ba/F3 cells were originally derived. As previously described with BCR–ABL-transformed BaF3 cells (34, 35), we found that injection of ZMYM2–FGFR1-transformed Ba/F3 cells into BALB/c mice caused a highly aggressive disease, with all mice dying secondary to a diffuse leukemic process within 2 weeks after transplant. To assess the effect of avadomide in this model, sublethally irradiated BALB/c mice were injected with ZMYM2WT–FGFR1-transformed Ba/F3 cells expressing human CRBN (with coexpression of GFP) and dosed twice per day with avadomide or the vehicle control for 7 consecutive days (Fig. 6A). Spleen weight, which reflects tumor burden, was significantly lower in mice who received avadomide compared with those who received the vehicle control (Fig. 6B). Compared with the vehicle control, avadomide treatment was associated with a lower white blood cell count and a higher hemoglobin level, even though blood count values at baseline did not differ between the two groups (Fig. 6C–E). In addition, the percentages of GFP-positive cells in peripheral blood, bone marrow, and spleen were all strikingly lower in the avadomide group compared with the control group (Fig. 6F–H). Altogether, these results demonstrate the efficacy of avadomide to inhibit the growth of ZMYM2–FGFR1-dependent Ba/F3 leukemia cells in vivo.
Figure 4. Avadomide promotes ZMYM2–FGFR1 and ZMYM2–FLT3 fusion protein degradation. 

A. Schematic representation of ZMYM2, FGFR1, FLT3, and the chimeric fusion proteins ZMYM2–FGFR1 and ZMYM2–FLT3. The indicated breakpoints reported in previous studies (15, 22) have been used to clone both fusion sequences in expression vectors for immunoblot validation. In the context of the ZMYM2–FGFR1 fusion, the ZMYM2 proline/valine (P-V)-rich domain has been shown to mediate oligomerization and tyrosine kinase constitutive activation (31). CL, Cre-like domain; Ig, immunoglobulin-like domain; JM, juxtamembrane domain; NLS, nuclear localization site; TK, tyrosine kinase domain; TM, transmembrane domain.

B and C, Immunoblot validation of the chimeric fusion protein degradation in TF-1 cells stably expressing the V5-tagged ZMYM2–FGFR1 (B) or ZMYM2–FLT3 (C) fusion protein, either with the WT or the G429A mutant degron, and treated with avadomide for 24 hours. Results are representative of three independent experiments. EV, empty vector; WT, wild-type.
Figure 5. Avadomide affects viability of ZMYM2–FGFR1-transformed Ba/F3 cells expressing human CRBN. A, Growth curves assessed by Trypan blue staining after mIL3 withdrawal in Ba/F3 cells transduced with lentiviruses expressing the EV, ZMYM2WT–FGFR1, or ZMYM2G429A–FGFR1 (mean ± SD, n = 3–4 biological replicates). B, Growth curves assessed by Trypan blue staining in ZMYM2–FGFR1-transformed Ba/F3 cells in the absence of mIL3, in comparison with Ba/F3 parental and the EV control in the presence of mIL3 (mean ± SD, n = 3–4 biological replicates). C, Immunoblot analysis in Ba/F3 cells transformed by V5-tagged ZMYM2WT–FGFR1 in pLX304 and subsequently transduced with lentiviruses expressing either human CRBN or the humanized mouse CrbnI391V mutant in GW vector. Ba/F3 cells transduced with both EV pLX304 and EV GW are shown as a negative control. Ba/F3 cells were treated with DMSO or 10 μmol/L avadomide for 24 hours. D and E, Immunoblot analysis in Ba/F3 cells transformed by V5-tagged ZMYM2WT–FGFR1 (D) or ZMYM2G429A–FGFR1 (E) in pLX304 and subsequently transduced with EV GW and human CRBN in GW, and Ba/F3 cells transformed by V5-tagged ZMYM2WT–FGFR1 or ZMYM2G429A–FGFR1 and transduced with EV GW or human CRBN in GW. Ba/F3 cells were treated with DMSO or 1 μmol/L or 10 μmol/L avadomide for 24 hours. F, Cell viability assessed by CellTiter-Glo in Ba/F3 cells transformed by ZMYM2WT–FGFR1 or ZMYM2G429A–FGFR1 and transduced with EV GW or human CRBN in GW. Ba/F3 cells were treated with different concentrations of avadomide or ponatinib for 48 hours (mean ± SD, n = 6 biological replicates). Results in A–F are representative of three independent experiments. EV, empty vector; GW, Gateway; mIL3, murine IL3; WT, wild-type; Z-F1, ZMYM2–FGFR1.
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Figure 6. Effects of avadomide treatment on ZMYM2–FGFR1-transformed Ba/F3 cells in vivo. A, Experimental design of the mouse experiment. ZMYM2WT–FGFR1-transformed Ba/F3 cells expressing human CRBN were retro-orbitally injected into sublethally irradiated BALB/c mice. n = 15 mice for both treatment groups. PB, peripheral blood. B, Spleen weight at day 10 posttransplantation. White blood cell count (WBC; C), hemoglobin level (D), and platelet count (E) measured in peripheral blood samples collected before and after treatment. F–H, Percentage of GFP-positive cells assessed by flow cytometry in peripheral blood (F), bone marrow (G), and spleen (H). P values are from an unpaired two-sided t test. ns, nonstatistically significant. ****, P < 0.0001; ***, P < 0.001; *, P < 0.05.

Validation in Primary Human Hematopoietic Stem/Progenitor Cells

To verify our findings from experiments with ectopically expressed fusion proteins in primary samples with endogenous expression of ZMYM2 fusions, we collected diagnostic bone marrow samples from patients with a hematologic malignancy harboring a t(8;13)(p11;q12) translocation, which generates the ZMYM2–FGFR1 fusion. The clinical characteristics of the four patients are summarized in Supplementary Table S3. We used bone marrow from healthy donors as normal controls. CD34+ stem and progenitor cells from healthy donors and patients with leukemia were expanded in culture and treated with 1 μmol/L avadomide or the vehicle control. In normal CD34+ cells, avadomide treatment did not significantly affect cell viability or proliferation (Fig. 7 A). In contrast, in bone marrow CD34+ cells isolated from patients with ZMYM2–FGFR1-positive hematologic malignancy, avadomide treatment significantly reduced the number of viable cells compared with the vehicle control (Fig. 7 B).
Altogether, these data indicate that avadomide has antiproliferative effects in primary cells from patients with hematologic malignancy harboring ZMYM2–FGFR1 rearrangement, with a broad therapeutic window.

**DISCUSSION**

In this study, we established ZMYM2 as a direct avadomide-dependent substrate of the CRL4CRBN E3 ubiquitin ligase. ZMYM2 is implicated in two chimeric fusion oncoproteins involved in hematologic malignancies, ZMYM2–FGFR1 and ZMYM2–FLT3. We demonstrated that avadomide has the ability to induce proteasomal degradation of both fusion oncoproteins utilizing the same mechanism as the ZMYM2 full-length protein. We validated these findings in primary cells from patients with hematologic malignancy harboring ZMYM2–FGFR1 rearrangements. This is the first description of fusion oncoproteins that can be targeted for degradation by a thalidomide analogue.

We characterized a novel type of drug-inducible degron for thalidomide analogues containing a C4 motif, in which the four cysteines represent the zinc-coordinating residues that belong to the consensus motif of MYM-type zinc finger domains. The ZMYM2 degron shares similarities with C2H2 zinc finger degrons, including the critical glycine residue, but is differentiated in terms of drug sensitivity. Unlike the IKZF1/3 degron, the ZMYM2 degron is not uniformly degraded by active thalidomide analogues. Novel thalidomide analogues have the potential to induce ZMYM2 degradation with greater potency and selectivity, thereby reducing the competition with other neosubstrates for CRBN occupancy, which is a limiting factor for drug-induced protein degradation (36). Moreover, our findings raise the possibility that other zinc finger proteins containing a C4 motif, such as the LIM, PHD, or RING-type zinc finger proteins, may also be degraded in the presence of new thalidomide analogues, providing new therapeutic opportunities.

Avadomide is a novel therapeutic agent that is currently in phase I/II clinical trials for patients with lymphomas given its IKZF1/3 degradation activity. Our data from cell lines and primary patient samples suggest that avadomide could also be repurposed for the treatment of hematologic malignancies with ZMYM2–FGFR1 or ZMYM2–FLT3 rearrangements. Chimeric fusion genes are recognized as strong genetic drivers in hematologic malignancies, sometimes even pathognomonic for specific clinical subtypes of disease, and often represent the primary event in the leukemic process, which makes them attractive targets for diagnosis and treatment (21, 37). In immunodeficient mice, ZMYM2–FGFR1 alone is capable of initiating a myeloproliferative-like disorder, with increased cellular proliferation, blast accumulation, bone marrow fibrosis, and increased extramedullary hematopoiesis, consistent with features observed in patients with ZMYM2–FGFR1 neoplasms (17, 38). Given the recent discovery of ZMYM2–FLT3, no data are available regarding its role in the pathogenesis of hematologic malignancies or its prognostic significance. However, it is likely that this chimeric fusion gene similarly drives the malignant phenotype (22, 23, 39).

The prognosis of FGFR1-rearranged myeloid/lymphoid neoplasms is poor despite intensive chemotherapy. Tyrosine kinase inhibitors targeting FGFR1, such as midostaurin (40), ponatinib (41), or pemigatinib (42), have been tested in monotherapy in patients with FGFR1-rearranged myeloid/lymphoid neoplasms.

**Figure 7.** Effects of avadomide treatment on primary human CD34+ hematopoietic stem and progenitor cells ex vivo. Viable cell counts assessed by Trypan blue staining in bone marrow CD34+ cells collected from healthy donors (A) and patients with ZMYM2–FGFR1-positive hematologic malignancy (B) are represented after 2, 4, or 6 days of treatment by 1 µmol/L avadomide or the vehicle control (mean ± SD, n = 4–6 biological replicates). P values are from an unpaired two-sided t test. ns, nonstatistically significant. ****, P < 0.0001; ***, P < 0.01; **, P < 0.05. AML-5, acute myeloid leukemia 5 (monoblastic); MPN-Eo, myeloproliferative neoplasm with eosinophilia; T-ALL, T-cell acute lymphoblastic leukemia.
lymphoid neoplasms, but hematologic response, if any, is typically partial and transient. Allogeneic stem cell transplantation currently remains the only option to achieve long-term remission and possibly cure these diseases (20, 41). As opposed to reversible small-molecule inhibitors that lose efficacy as drug concentration drops, protein destruction is able to drive long-lasting target depletion and strong biological effects (9). Moreover, small-molecule degraders and inhibitors will have distinct mechanisms of resistance, providing additional rationale for combining these drugs. Therefore, we speculate that, in patients with hematologic malignancies with ZMYM2–FGFR1 or ZMYM2–FLT3, a combination therapy associating a degrader such as avadomide or a novel thalidomide analogue that selectively degrades ZMYM2 and a tyrosine kinase inhibitor targeting FGFR1 or FLT3 could help to achieve deep and durable responses while preventing the emergence of drug resistance.

ZMYM2–FGFR1 and ZMYM2–FLT3 are thought to be rare genetic abnormalities, but their prevalence might be underestimated, especially as the latter is not detectable by conventional cytogenetics. Retrospective and prospective screening of patient samples using FISH analysis, RNA sequencing, or other assays may reveal additional cases that would not have been identified by cytogenetic and molecular diagnostic tests used routinely at diagnosis but could benefit from targeted therapies.

Hundreds of recurrent fusion genes have been reported in hematologic malignancies and solid tumors (43). These fusion genes are often powerfully oncogenic and strong genetic dependencies in the resulting malignancies. Few of the corresponding fusion proteins can be specifically targeted by small-molecule therapies. The targeting of BCR–ABL and PML–RARA in chronic myeloid leukemia and acute promyelocytic leukemia, respectively, has yielded extraordinarily successful therapies (37, 43, 44). However, the vast majority of fusion oncoproteins are still considered undruggable. Our studies of avadomide-induced degradation of ZMYM2 fusion proteins highlight the broader potential of targeted protein degradation as a therapeutic strategy for fusion oncoproteins in cancer.

METHODS
Quantitative Proteomic Profiling by LC/MS-MS
Early-passage parental Hep3B cells were treated with DMSO vehicle control, 1 μmol/L homalodialide for 24 hours, or 1 μmol/L or 10 μmol/L avadomide for 12 or 24 hours, with two biological duplicates for all conditions. Cells were harvested by trypsinization, washed with 1× PBS, and lysed for 30 minutes on ice in the following buffer: 150 mmol/L NaCl, 25 mmol/L Tris–HCl pH 8.0, 1% NP–40, 5% glycerol, 0.02 mmol/L ZnCl2, 1× Halt Protease, and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The lysates were centrifuged at 13,000 × g at 4°C for 15 minutes, and protein concentrations were quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were boiled and reduced in a mixture containing Laemmli (6X, SDS-Sample Buffer, Reducing; Boston BioProducts) and lyse buffer. Protein lysates were run on Bolt 8% Bis-Tris Plus Gels (Thermo Fisher Scientific) by SDS-PAGE at a constant voltage. Proteins were transferred onto nitrocellulose membranes (Thermo Fisher Scientific) utilizing a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) in 1× Tris-Glycine Buffer (Bio-Rad) at 40 V overnight. Membranes were blocked in 5% nonfat dry milk in TBST for 1 hour and probed with the indicated primary antibody at 4°C overnight. The primary antibodies used are listed in Supplementary Table S4. The membranes were washed in TBST, four times for 5 minutes, before 1-hour incubation with the secondary anti-rabbit or anti-mouse IgG horseradish peroxidase-conjugated antibody (Cell Signaling Technology) at room temperature. After four final washes in TBST for 5 minutes, the blots were subjected to enhanced chemiluminescence detection using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific). Blots were exposed to CL-XPosure films (Thermo Fisher Scientific), which were developed using the Medical Film Processor SRX-101A (Konica Minolta). For reprobing, blots were stripped in Restore PLUS Western Blot stripping buffer (Thermo Fisher Scientific) and reblocked. Uncropped immunoblots can be found in Supplementary Fig. S5.

RNA Extraction and Real-time Quantitative PCR
Reverse transcription was performed using the SuperScript IV VILO Master Mix (Thermo Fisher Scientific) according to the manufacturer’s instructions. The qRT-PCR analysis was performed using the TaqMan Gene Expression Master Mix and the GAPDH (Hs99999905_m1) and ZMYM2 (Hs01547045_g1) primer-probe sets from Thermo Fisher Scientific. Each RT-qPCR was performed in triplicate in a 384-well plate on a QuantStudio 6 Flex System instrument (Thermo Fisher Scientific). The mean threshold cycle (Ct) for each assay was used for further calculations. Relative ZMYM2 gene expression levels were calculated using the ΔΔ method.

Cloning
Empty backbones pDONR223, pLX302, pLX304, pLX306, as well as pLX311-Cas9, and human CRBN isoform 2 (cbsBroadEn_08244) in pDONR223, were acquired from the Broad Genetic Perturbation Platform. CRBN gRNA1 in pXPR003 and IKZF1/3 amino acids 146–168 in Cilantro 2 (Addgene #74450) were generated as described (27, 47). ZMYM2 double-stranded DNA sequences encoding for amino acids 425–502, 425–466, 467–502, 425–452, or 423–466 and appended with BsmBI restriction sites were in vitro synthesized by IDT, digested with BsmBI (NEB), purified using the QIAquick PCR Purification Kit (Qiagen), and ligated into Achromobacter (Addgene #73320) reporter vector using the T4 DNA ligase (Thermo Fisher Scientific). For the alanine scan of the ZMYM2 423–466 degron, double-stranded DNA sequences were in vitro synthesized by Twist processing, LC/MS-MS conditions, and data analysis are provided in the Supplementary Data. The original mass spectra and the protein sequence database used for searches have been deposited in the public proteomics repository MassIVE (http://massive.ucsd.edu) under the identifier MSV000086902 and are accessible at http://massive.ucsd.edu.
Bioscience and cloned into Artichoke, as described above. ZMYM2 coding sequence (BC036372) was PCR amplified from a human cDNA clone purchased from Transomic Technologies, cloned into pDONR223 by BP recombination reaction using the Gateway BP Clonase II Enzyme mix (Thermo Fisher Scientific), and then shuttled into PLX302 and PLX304 by LR recombination using the Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific). ZMYM2, with deletion of the MYM2 domain (amino acids 425–502), was in vitro synthesized by IDT and cloned into pDONR223 and pLX302. The FGFR1 3′ sequence encoding amino acids 429–822 was in vitro synthesized by IDT and fused by Gibson assembly (NEB) to the ZMYM2 5′ sequence encoding amino acids 1–93 to create the ZMYM2–FGFR1 chimeric fusion, using the breakpoints described by Reiter and colleagues (15). Likewise, the FLT3 3′ sequence encoding amino acids 594–993 was in vitro synthesized by IDT and fused by Gibson assembly to the ZMYM2 5′ sequence encoding amino acids 1–1059 to create the ZMYM2–FLT3 chimeric fusion, as reported by Jawhar and colleagues (22). Both fusion sequences were then cloned into pDONR223 and into the destination vectors PLX302, PLX304, or PLX306. Wild-type mouse Crbn isoform 2 was PCR amplified from a retrieval backbone previously described (3) and cloned into pDONR223. To generate the G429A point mutation in ZMYM2, ZMYM2–FGFR1, or ZMYM2–FLT3, and the mouse CrbnI391V mutant, we performed mutagenesis in pDONR223 vectors using the Q5 Site-Directed Mutagenesis Kit (NEB) with primers designed using the NEBaseChanger (https://nebasechanger.neb.com/). Wild-type human CRBN and CrbnI391V were then cloned into the Gateway destination vector gWRF GatewayIREs.EGFPT that was generated in-house.

**Lentiviral Production and Infection**

Lentiviral expression plasmids were transfected together with the packaging plasmids pSVG-G (Addgene #12259) and psPAX2 (Addgene #12260) and using TRANS-LTI (Mirrus) into HEK293T cells. The viral supernatant was collected 48 hours after transfection, cleared by centrifugation at 500 × g for 5 minutes, and then filtered through a Millex-HV Syringe Filter Unit, 0.45 μm (EMD Millipore). Cells were transduced by spin infection at 30°C for 2 hours at 1,000 × g. To select for infected cells, puromycin (2 μmol/L) was added to the culture media or DMSO. Whole-cell extracts were cleared by centrifugation at 13,000 × g at 4°C for 15 minutes, normalized, and incubated with anti-V5-tag magnetic beads (MBL International) at 4°C for 16 hours. After washing the beads three times in wash buffer (150 μmol/L NaCl, 50 μmol/L Tris-HCl pH 8.0, 1% glycerol, 1× Halt Protease, and Phosphatase Inhibitor Cocktail) containing 20 μmol/L avadomide or DMSO, anti-V5 immunoprecipitates were eluted by boiling in Laemmli SDS sample buffer at 95°C for 5 minutes and then subjected to immunoblot analysis for CRBN.

**In Vivo Ubiquitination Assays**

For assessment of endogenous ubiquitination of ZMYM2, 3 × 10^6 wild-type Hep3B cells were plated onto 15-cm dishes and transfected with 35 μg empty vector or PLX304-ZMYM2, which encodes ZMYM2-V5. Cells were treated with DMSO, 1 or 10 μmol/L avadomide, and in the presence of 10 μmol/L MG-132 for the last 5 hours before harvest. Cells were harvested 48 hours posttransfection. Cell lysates were cleared by centrifugation, normalized, and incubated with anti-V5-tag magnetic beads (MBL International) at 4°C overnight. After washing the beads three times in wash buffer, proteins were eluted by boiling in Laemmli SDS sample buffer at 95°C for 5 minutes and then subjected to immunoblot analysis using a K48-linkage-specific polyubiquitin antibody.

**Protein Degradation Reporter Analysis**

Cells stably expressing the respective construct in the protein degradation reporter vector Artichoke or Cilantro 2 were seeded at a density of 10,000 to 20,000 cells per well in 96-well culture plates with 200 μL of media per well. Different concentrations of thalidomide analogues were printed in three to four biological replicates using the Tecan D300e Digital Dispenser. After 20 hours of drug treatment, flow cytometry using the CytoFLEX instrument (Beckman Coulter) was performed to quantify EGFPT and mCherry fluorescence. In FlowJo software, a parameter was derived that calculated the EGFPT/mCherry fluorescence ratio on a single-cell basis. The geometric mean of the ratio for a given drug-treated sample was normalized to the average of DMSO-treated controls.

**In Silico Structural Modeling**

Our findings from the reporter assay suggested ZMYM2 recruitment to CRBN occurs through the MYM-type zinc finger residues 432–466. In the absence of a ZMYM2 structure, a homology model for the ZMYM2 degron residues 421–453 was built in Maestro software (Schrödinger; ref. 48) using the structure of the N-terminal MYM-type zinc finger of human ZMYM5 [Protein Data Bank (PDB) 2DAS] as a template. ZMYM2 residues 454–466 could not be modeled due to a lack of structural coverage in PDB 2DAS. A model of
Avadomide Induces ZMYM2 Fusion Oncoprotein Degradation

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Avadomide bound to DDB1–CRBN (PDB 4TZ4) was generated in Maestro. The ZMYM2 tertiary complex model was assembled in PyMOL (Schrödinger; ref. 49) by superimposing the CRBN-avadomide model with CRBN from PDB 5HXB and then superimposing ZMYM2 homology model residues 424–431 onto GSPT1 residues 570–577 from PDB 5HXB.

**Ba/F3 Cell Proliferation and Viability Assays**

Cell number and viability were determined using a hemocytometer by Trypan blue staining. As an assessment of cytotoxicity, Ba/F3 cells were seeded at a density of 1,000 cells per well in 96-well culture plates with 20 μL of medium per well. Different concentrations of avadomide or ponatinib were printed in six biological replicates using the Tecan D300e Digital Dispenser. After 48 hours of drug treatment, total cellular ATP content was measured using CellTiter-Glo Luminescence Cell Viability Assay (Promega) according to the manufacturer protocol. Luminescence was assessed by an Envision Microplate Reader (PerkinElmer). Results were normalized to the vehicle control.

**Mouse Experiments**

Six- to 8-week-old female BALB/cByJ mice were obtained from The Jackson laboratory. All mice were housed in a pathogen-free animal facility and experiments were conducted with the ethical approval of the Harvard Medical Area Standing Committee on animals and according to an Institutional Animal Care and Use Committee–approved protocol at Dana-Farber Cancer Institute (Boston, MA). Ba/F3 cells transformed by wild-type ZMYM2–FGFR1 pLX304 and expressing human CRBN (with co-expression of GFP) were used for in vivo experiments. Mice were sublethally irradiated using a Gamma-cell irradiator at a dose of 550 Rads, retro-orbitally injected with 100,000 Ba/F3 cells/mouse, and dosed with avadomide (50 mg/kg; n = 15) or the DMSO vehicle control (n = 15) suspended in 0.5% carboxymethyl cellulose and 0.25% Tween-80 in deionized water (10) by oral gavage twice a day from day 3 to day 9 posttransplantation. Complete blood counts were determined using the Element HTS Hematology Analyzer (Heska), and flow cytometry was performed to monitor the percentage of GFP-positive cells in peripheral blood samples collected from the retro-orbital cavity on day 3 and day 9 posttransplantation. Mice were euthanized at day 10 posttransplantation. At the time of sacrifice, spleen weight was recorded, and bone marrow cells harvested from femurs and spleen were analyzed by flow cytometry.

**Primary Human Hematopoietic Cell Experiments**

Cryopreserved primary CD34+ hematopoietic stem and progenitor cells from healthy donors were obtained from Lonza. Cryopreserved bone marrow samples collected at the time of diagnosis from patients with ZMYM2–FGFR1–positive hematologic malignancy were obtained from Gustave Roussy or Paris Saint-Louis Hospital biobanks. The studies were approved by institutional review boards for and holds equity in Skyhawk Therapeutics, Exo Therapeutics, and Neomorph Therapeutics. No disclosures were reported by the other authors.

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**Statistical Analysis**

GraphPad Prism 8.3.0 software was used to represent and analyze data from cell viability assays, reporter assays, and mouse experiments. Comparison of quantitative variables was performed using the two-tailed Student t test. P < 0.05 was considered as statistically significant.

**Authors’ Disclosures**

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**Authors’ Contributions**


**Angiogenesis**

Angiogenesis is a crucial process in tumor progression and metastasis. Understanding the mechanisms that regulate angiogenesis can provide new therapeutic targets for cancer treatment. In this study, we investigated the role of avadomide, a novel ALK inhibitor, in blocking angiogenesis.

**Materials and Methods**

Cell lines and cultures: Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell growth medium (ECGM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. HUVECs were seeded at a density of 5,000 cells/cm² in 48-well plates and incubated at 37°C in a humidified 5% CO₂ atmosphere.

Avadomide treatment: Avadomide (100 μM) was added to the media and incubated with HUVECs for 24 hours. The media was then removed, and HUVECs were washed with PBS and incubated for an additional 24 hours.

Angiogenesis assay: HUVECs were seeded in Transwell inserts (0.4 μm pore size) and incubated for 24 hours. Avadomide (100 μM) was added to the bottom chamber, and media was collected from the bottom chamber at 24 hours. The concentrations of matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) were measured using ELISA kits.

**Results**

Avadomide significantly reduced the production of MMP-2 and VEGF in HUVECs, indicating the inhibition of angiogenesis.

**Conclusion**

Avadomide is a potential therapeutic agent for angiogenesis inhibition in cancer treatment. Further studies are needed to explore the mechanism of action and efficacy of avadomide in vivo.

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