AMG 176, a Selective MCL1 Inhibitor, Is Effective in Hematologic Cancer Models Alone and in Combination with Established Therapies

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ABSTRACT
The prosurvival BCL2 family member MCL1 is frequently dysregulated in cancer. To overcome the significant challenges associated with inhibition of MCL1 protein–protein interactions, we rigorously applied small-molecule conformational restriction, which culminated in the discovery of AMG 176, the first selective MCL1 inhibitor to be studied in humans. We demonstrate that MCL1 inhibition induces a rapid and committed step toward apoptosis in subsets of hematologic cancer cell lines, tumor xenograft models, and primary patient samples. With the use of a human MCL1 knock-in mouse, we demonstrate that MCL1 inhibition at active doses of AMG 176 is tolerated and correlates with clear pharmacodynamic effects, demonstrated by reductions in B cells, monocytes, and neutrophils. Furthermore, the combination of AMG 176 and venetoclax is synergistic in acute myeloid leukemia (AML) tumor models and in primary patient samples at tolerated doses. These results highlight the therapeutic promise of AMG 176 and the potential for combinations with other BH3 mimetics.

SIGNIFICANCE: AMG 176 is a potent, selective, and orally bioavailable MCL1 inhibitor that induces a rapid commitment to apoptosis in models of hematologic malignancies. The synergistic combination of AMG 176 and venetoclax demonstrates robust activity in models of AML at tolerated doses, highlighting the promise of BH3-mimetic combinations in hematologic cancers. Cancer Discov; 8(12): 1582-97. © 2018 AACR.

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INTRODUCTION

The evasion of apoptosis is a hallmark of cancer, sustaining tumor growth, survival, and resistance to a broad spectrum of anticancer therapeutics (1). Dysregulation of the B-cell lymphoma-2 (BCL2) family of proteins is frequently responsible for this circumvention of programmed cell death (2). The BCL2 family of proteins is comprised of antiapoptotic members, including BCL2, MCL1, and BCL-xL, and proapoptotic members, which are further divided into two groups: the BH3-only proteins, for example, BIM and BAD, and the downstream apoptotic effectors BAK and BAX (3). The dynamic interplay between these proteins is integral to controlling the apoptotic threshold of cells, with the BH3 domain of proapoptotic family members binding to a hydrophobic groove on the surface of their antiapoptotic counterparts (4). Small-molecule BH3 mimetics, the most advanced of which is the BCL2 inhibitor venetoclax, compete for binding to this groove, enabling endogenous BH3-only proapoptotic proteins to activate downstream components of the pathway (5).

The impressive clinical activity of venetoclax in chronic lymphocytic leukemia has provided validation for BH3 mimetics in cancer (6). However, more modest response rates in multiple myeloma and acute myeloid leukemia (AML) suggest involvement of other antiapoptotic family members in these settings (7, 8). Studies involving genetically engineered mouse models, RNAi, and CRISPR/Cas9 genome editing have implicated MCL1 in promoting the survival of multiple hematologic malignancies, including multiple myeloma, AML, and MYC-driven lymphomas, highlighting the potential for MCL1 as a therapeutic target in these indications (9–15).

The shallow BH3-domain binding pocket in MCL1, along with the high-affinity interactions with its binding partners, has made the development of potent and selective MCL1 inhibitors with suitable drug-like properties challenging. However, recent success has been reported with the small molecule S63845 (16).

We report the discovery of AMG 176, a first-in-class orally bioavailable MCL1 inhibitor in clinical development for hematologic malignancies (ClinicalTrials.gov; NCT02675452).
Using structure-based design, we optimized a series of spiro- macrocyclic molecules integrating conformational restriction as a guiding principle throughout the optimization process (17). In hematologic cancer cell lines, this class of MCL1 inhibitors induced a rapid commitment toward apoptosis at nanomolar concentrations following exposure to drug for as little as 30 minutes. Discontinuous oral administration of AMG 176 inhibited the growth of human AML and multiple myeloma tumor xenografts at tolerated doses. Furthermore, in contrast to published reports with S63845, where little to no effect was observed on white blood cell counts following intravenous administration of S63845 at the maximum tolerated dose, oral administration of AMG 176 resulted in dose-dependent reductions in B cells, monocytes, and neutrophils, highlighting their potential as pharmacodynamic endpoints of MCL1 inhibition (16). Finally, we show that the combination of AMG 176 and venetoclax was synergistic in primary AML patient samples and demonstrated robust activity in AML xenograft models at tolerated doses. These data warrant further evaluation of AMG 176 in the clinical setting and highlight the promise of combined MCL1 and BCL2 inhibition as a means of achieving maximal clinical benefit with BH3 mimetics in hematologic cancers.

RESULTS

AMG 176 Is a Potent and Selective MCL1 Inhibitor

A successful strategy for inhibiting protein–protein interactions (PPI) is to identify weakly bound small-molecule fragments and improve their affinity by increasing their size to gain additional interactions with the target protein (18). Although this approach improves affinity, it often results in molecules with poor selectivity and pharmacokinetic properties, such as low oral bioavailability, presumably due to their large size and high flexibility (19). Guided by X-ray structure and small-molecule conformational analysis, we approached the optimization of inhibitors of MCL1 with the strategy of conformational restriction to reduce nonbinding conformations (17). Based on the hypothesis that high levels of nonbinding conformations increase the likelihood of poor selectivity and pharmacokinetic properties, we successfully used conformational restriction as a guiding principle for the optimization of these PPI inhibitors.

Compound 1 was identified as a racemate (IC$_{50}$ = 3.4 μmol/L) from a screen of a 248,090-compound library for disruption of the MCL1/BIM interaction (Fig. 1A). Separation of the enantiomers (2 and 3 in Fig. 2A) and expansion of the central 6-membered heterocyclic ring to a 7-membered heterocycle provided compound 4 (IC$_{50}$ = 0.3 μmol/L). Co-crystallization of 4 with MCL1 revealed a cryptic binding pocket not present in the cocrystal structure of MCL1 when bound to the BIM peptide (Fig. 1A–C; vide). PDB validation reports 9100015620 and 9100015541; Supplementary Table S1; ref. 20). Examination of the MCL1/4 structure revealed the near coplanarity of the stereogenic hydrogen and the ortho-chlorine, prompting an exchange of these atoms for an ethylene unit that could better fill the cryptic pocket and engender conformational restriction by constraining the rotation of the aryl ring (Fig. 1A). Gratifyingly, spirocyclic 5 showed improved biochemical potency (IC$_{50}$ = 0.04 μmol/L). In an effort to maintain the ionic interaction between the carboxylic acid of 2 and Arg 263 of MCL1 while introducing an additional vector for derivatization, compound 6 was synthesized. A nuclear magnetic resonance (NMR) structure of compound 6 complexed with MCL1 showed a binding conformation of the benzyl acyl sulfonamide where the phenyl ring was in close proximity to the four-membered ring, suggesting conformational restriction could again be employed to form a macrocyclic ring (PDB validation report 9100015681). Combination of the conformationally restricted macrocyclic and spirocyclic rings provided the high-affinity MCL1 inhibitor, compound 7 (IC$_{50}$ = 0.01 μmol/L). The binding conformations of compounds 5 and 7, when complexed with MCL1, suggested that fusion of a trans-cyclobutane onto the macrocycle would generate additional hydrophobic contacts and further conformationally restrict the macrocycle (PDB validation reports 9100015557 and 9100015544; Supplementary Table S1). Following this hypothesis resulted in the synthesis of compound 8, which showed a significant increase in potency (Ki = 0.00014 μmol/L). The X-ray crystal structure of compound 8 complexed with MCL1 along with molecular modeling revealed that the conformation of 8 observed in complex with MCL1 was the fifth most populated conformation, representing only 12% of the total conformational ensemble (Fig. 1D; PDB validation report 9100015543; Supplementary Table S1). Analysis of compound 8 provided an opportunity to further conformationally restrict the macrocycle by installation of a trans-olefin between carbons 7 and 8, yielding compounds 9 and 10 (Ki = 0.00004 μmol/L and 0.00005 μmol/L, respectively). Compound 10 (AM-8621) had a suitable potency and selectivity profile to serve as a tool MCL1 inhibitor for characterizing the mechanism of action of MCL1 inhibition in vitro. The improvement in potency of compound 9 was presumably due to conformational restriction, where now the observed conformation of compound 9 in complex with MCL1 was the most abundant conformation (45%; Fig. 1E; PDB validation report 9100015542). Moreover, installation of the olefin reduced the number of energetically accessible conformations within 3 kcal/mol from 22 (compound 8) to 8 (compound 9). Compounds 9 and 10 had short half-lives and poor oral bioavailability (Fig. 1F). Simple methylation of the alcohol led to a series of compounds with improved pharmacokinetic profiles, including increased oral bioavailability, which ultimately provided compound 11 or AMG 176, the first selective, orally bioavailable MCL1 inhibitor to advance into human clinical trials, validating our strategy of optimization guided by conformational restriction (video; ClinicalTrials.gov; NCT02675452).

Disruption of MCL1 Interactions Induces Apoptosis

AMG 176 and the related analogue AM-8621 exhibit picomolar affinity for human MCL1, approximately 1,000-fold reduced affinity toward murine MCL1, and minimal binding affinity toward BCL2 and BCL-xl (Fig. 1A). The ability of the tool compound, AM-8621, to disrupt the interaction between MCL1 and BAK was evaluated in HEK293M cells using a split-luciferase complementation assay (IC$_{50}$ = 43 nmol/L; Fig. 2A; ref. 21). AM-8621 also disrupted the interaction between MCL1 and BIM in a co-complex immunoassay, exhibiting dose- and time-dependent inhibition in the non–small cell lung cancer (NSCLC) cell line A427 (Fig. 2B). A427 cells were selected for these studies because of their appreciable MCL1:BIM co-complex levels and relative insensitivity to AM-8621 treatment.
Figure 1. Optimization of chemical matter to AMG 176. A, X-ray structure–based optimization of high-throughput screening hit to clinical candidate AMG 176. X-ray structure suggested spirocyclic fusion (circled). B, X-ray crystal structure of MCL1 bound to BIM (20). C, X-ray structure of MCL1 bound to AM-8621 reveals cryptic binding pocket (PDB validation report 9100015541). D, Quantum mechanical–derived conformational ensemble of 8 within 3 kcal/mol depicted as Boltzmann distribution. Binding conformation shown in green. Broken bars represent multiple conformations. PCM, polarizable continuum model. E, Quantum mechanical–derived conformational ensemble of 9 within 3 kcal/mol depicted as Boltzmann distribution. Binding conformation shown in green. Broken bars represent multiple conformations. F, Pharmacokinetic properties of 9, 10, and 11. Species refers to the species of animal in which the pharmacokinetic data were acquired.
We next evaluated the effect of AM-8621 on MCL1 protein levels (22). The AM-8621–insensitive multiple myeloma cell line U266B1 was selected for these experiments so that changes in MCL1 protein levels would not be influenced by treatment-induced effects on cell viability. A dose-dependent induction of MCL1 protein was observed following compound treatment (Fig. 2C). Although independent of changes in transcription (Supplementary Fig. S1), this increase was at least partially driven by an extended protein half-life (Fig. 2D). Consistent with rapid target engagement, elevated MCL1 protein was detected within 15 minutes of treatment initiation. MCL1 levels returned to baseline 4 hours after washout of AM-8621, confirming the reversibility of compound binding (Fig. 2E). This induction of MCL1 protein following AM-8621 treatment was not limited to U266B1 cells; similar observations were made in several additional cell lines (including A427, MV-4-11, and NCI-H1568; Supplementary Fig. S2).

AM-8621 was then tested for its ability to activate the intrinsic apoptosis pathway. Treatment with AM-8621 increased activated BAK levels, a proximal downstream effector of MCL1, in a panel of AM-8621–sensitive cell lines (OPM-2, multiple myeloma; MV-4-11, AML; MOLM13, AML; and Ramos, Burkitt lymphoma) but not in the AM-8621–insensitive cell line U266B1 (multiple myeloma; Fig. 3A). Together with the liberation of BH3 domain–containing proteins, BAK activation represents a key prelude to the induction of downstream components of the intrinsic apoptosis pathway, including caspase 3 and 7 (5). Highly AM-8621–sensitive cell lines (OPM-2, MV-4-11, MOLM13, and Ramos)
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Effects of AM-8621 treatment (1 μmol/L for 2 hours) on activated BAK in AM-8621-sensitive (OPM-2, MV-4-11, MOLM13, and Ramos) and AM-8621-insensitive (U266B1) cell lines as measured in a flow cytometry-based BAK activation assay.

Figure 3. AM-8621 induces a rapid commitment toward apoptosis and loss of viability in sensitive cell lines. A, Effects of AM-8621 treatment (1 μmol/L for 2 hours) on activated BAK in AM-8621-sensitive (OPM-2, MV-4-11, MOLM13, and Ramos) and AM-8621-insensitive (U266B1) cell lines as measured in a flow cytometry-based BAK activation assay. B, Caspase activity and effects on viability following treatment with AM-8621 in OPM-2, MV-4-11, MOLM13, and Ramos cells. Effects on cell viability and caspase activity were measured by CellTiter-Glo and Caspase-Glo, respectively. Mean and ±SD from n = 4.

Viability analysis of OPM-2, MV-4-11, MOLM13, and Ramos cells treated with AM-8621 for 24 hours. Effects on cell viability were measured by CellTiter-Glo. Mean and ±SD from n = 4.

Viability analysis of AMO1, H929, and OPM-2 parental and BAX−/−/BAK−/− cell lines treated with AM-8621 for 24 hours. Effects on cell viability were measured by CellTiter-Glo. Mean and ±SD from n = 3.

Figure 3. AM-8621 induces a rapid commitment toward apoptosis and loss of viability in sensitive cell lines. A, Effects of AM-8621 treatment (1 μmol/L for 2 hours) on activated BAK in AM-8621-sensitive (OPM-2, MV-4-11, MOLM13, and Ramos) and AM-8621-insensitive (U266B1) cell lines as measured in a flow cytometry-based BAK activation assay. B, Caspase activity and effects on viability following treatment with AM-8621 in OPM-2, MV-4-11, MOLM13, and Ramos cells. Effects on cell viability and caspase activity were measured by CellTiter-Glo and Caspase-Glo, respectively. Mean and ±SD from n = 4.

Viability analysis of OPM-2, MV-4-11, MOLM13, and Ramos cells treated with AM-8621 for 24 hours. Effects on cell viability were measured by CellTiter-Glo. Mean and ±SD from n = 4.

Viability analysis of AMO1, H929, and OPM-2 parental and BAX−/−/BAK−/− cell lines treated with AM-8621 for 24 hours. Effects on cell viability were measured by CellTiter-Glo. Mean and ±SD from n = 3.

exhibited caspase 3 and 7 activation within 1 to 4 hours of treatment. Reductions in cell viability were observed shortly thereafter, demonstrating a near-maximal response within 8 hours of treatment initiation (Fig. 3B). AM-8621 washout studies revealed a further dependence on MCL1 in these lines. Although a limited effect on OPM-2 viability was observed 1 hour after treatment initiation, subsequent washout of AM-8621, followed by incubation in the absence of drug for 23 hours, resulted in >80% reduction in viability approaching that achieved with 24 hours of continuous treatment. Similar observations were made in Ramos cells following 30 minutes of treatment, and to a lesser extent in MV-4-11 and MOLM13 cells following 2 hours of treatment (Fig. 3C). To confirm the on-mechanism activity of this class of MCL1 inhibitors, BAX−/−/BAK−/− AMO1, H929, and OPM-2 cells were treated with AM-8621 and compared with parental cell lines (9). In
contrast to the parental cell lines, no effect on viability was observed with any of the BAX−/− BAK−/− lines, providing compelling evidence for the on-target MCL1-mediated activity of these compounds (Fig. 3D).

Hematologic Cancer Cell Lines Are Sensitive to MCL1 Inhibition

To identify those tumor types with greatest sensitivity to MCL1 inhibition, AM-8621 was profiled against a panel of 952 tumor cell lines (ref. 23; Fig. 4A). Cell lines derived from hematologic malignancies exhibited greater sensitivity to AM-8621 than did solid tumor lines (P < 1 × 10−13, Fisher exact test). The hematologic indications exhibiting greatest sensitivity included multiple myeloma (P = 0.006), AML (P = 1 × 10−5), and B-cell lymphoma (P < 1 × 10−6), with subsets of ALL and Burkitt lymphoma also exhibiting dependency on MCL1 (Fig. 4B; Supplementary Table S2). Among solid tumor cell lines, breast cancer lines demonstrated the greatest sensitivity to AM-8621 (P = 0.001).

To understand the dependency of hematologic cancer cell lines on specific prosurvival BCL2 family members, expanded panels of multiple myeloma, AML, and diffuse large-B-cell lymphoma (DLBCL) cell lines were assessed for sensitivity to AM-8621 and the BCL2-selective inhibitor venetoclax. In agreement with results from the initial screen, dependency on MCL1 was observed in a subset of cell lines (Fig. 4C). Most notably, multiple myeloma cell lines relied predominantly on MCL1 for survival, whereas AML and DLBCL cell lines were more heterogeneous, exhibiting sensitivity to selective inhibition of MCL1, BCL2, or both (Fig. 4D).

Low BCL-xL and High BAK Expressions Predict for Sensitivity to MCL1 Inhibition

To identify predictive biomarkers of response to AM-8621, we assessed the relationship between sensitivity and genomic features within the 952 tumor cell lines. A multivariate linear regression analysis (elastic net) was used to capture determinants of response to AM-8621 (23). Among all genomic features included in the model, expression or copy-number variation of a small number of genes (n = 165) was predictive of response across all cell lines (Supplementary Table S3). Strikingly, high BCL-xL (BCL2L1) expression was the strongest predictor of resistance, whereas, conversely, high expression of BAK (BAK1) was the strongest predictor of sensitivity. MCL1 expression was not predictive. The identification of BCL-xL expression as a resistance feature was confirmed by the strong correlations observed between AM-8621 sensitivity and BCL-xL transcript and protein expression in the expanded panel of multiple myeloma cell lines, with high BCL-xL expression again being associated with resistance to AM-8621 (Fig. 4E; Supplementary Fig. S3). The identification of BCL-xL and BAK, both members of the BCL2 protein family, as the strongest predictors of resistance/response provides further evidence supporting the on-mechanism activity of AM-8621.

AMG 176 Is Efficacious in Multiple Myeloma and AML Xenograft Models and Well Tolerated in Human MCL1 Knock-In Mice

To characterize the kinetics of apoptosis induction in vivo, we used the clinical-stage molecule AMG 176, given its superior pharmacokinetic properties over AM-8621. Dose-dependent activation of the intrinsic apoptosis pathway in OPM-2 xenografts, as measured by activated BAK, cleaved caspase-3, and cleaved PARP, was detected as early as 2 hours after oral administration of AMG 176 (Fig. 5A), with sustained cleaved PARP and activated BAK detectable through 12 hours and cleaved caspase-3 through 24 hours. Immunohistochemistry analysis revealed a similar dose-dependent increase in cleaved caspase-3 (Supplementary Fig. S4).

The ability of AMG 176 to rapidly induce apoptosis in pharmacodynamic assays suggested that it may be efficacious when administered using a discontinuous schedule. To test this hypothesis, mice harboring subcutaneous OPM-2 xenografts were treated with AMG 176 twice weekly and compared with mice treated daily. Discontinuous doses of 30 and 60 mg/kg were selected based on their ability to induce apoptotic markers in pharmacodynamic assays. Twice-weekly oral administration of AMG 176 at a dose of 30 mg/kg achieved 54% tumor growth inhibition (TGI) relative to vehicle, whereas a dose of 60 mg/kg achieved 21% tumor regression relative to initial tumor volume. Daily administration of AMG 176 achieved 84% TGI and 100% regression, respectively (Fig. 5B). Similarly, once-weekly oral administration of AMG 176 at doses of 50 and 100 mg/kg achieved 97% TGI and 70% regression, respectively (Fig. 5C). We next assessed the activity of AMG 176 in the MOLM13 luciferase-labeled orthotopic model of AML, in which tumor cells have engrafted in the bone marrow of mice. Twice-weekly oral administration of AMG 176 at 30 or 60 mg/kg resulted in significant dose-dependent inhibition of tumor burden as assessed by whole-body luminescence [28% and 69% reduction in bioluminescence imaging (BLI), respectively; Fig. 5D].

Several conditional knock-out studies have documented the dependency of B cells, monocytes, neutrophils, and/or their progenitors on MCL1 for survival, suggesting these cell types may serve as pharmacodynamic markers of MCL1 inhibition (24–27). Furthermore, the failure of AMG 176 to inhibit mouse MCL1 limits our understanding of the relationship between tolerability and effects on normal cell types at efficacious doses. To further investigate these relationships, we generated a human MCL1 knock-in mouse, replacing the Mcl1 gene with its human ortholog (Supplementary Fig. S5). Ex vivo treatment of splenocytes with AM-8621 for 6 hours resulted in caspase 3 activation and reduced viability in B cells derived from human MCL1 knock-in, but not wild-type, mice (Supplementary Fig. S6). These effects translated in vivo where oral administration of AMG 176 at 30 and 60 mg/kg resulted in dose-dependent decreases in B cells, monocytes, and neutrophils in the blood (Fig. 5E; Supplementary Fig. S7). Similar reductions were observed in bone marrow, highlighting the ability of AMG 176 to distribute into tissues. Broader complete blood count (CBC) analysis revealed additional effects on eosinophils, basophils, and reticulocytes (Supplementary Fig. S8). No evidence of overt systemic toxicity was observed in either AMG 176 treatment group as determined by changes in body weight (Supplementary Fig. S9).
Figure 4. Cell lines from hematologic cancers, including multiple myeloma (MM), AML, and DLBCL, exhibit a strong dependency on MCL1 for survival. A, Profile of response to AM-8621 for 952 tumor-derived cell lines grouped by cancer subtype. SCC, squamous cell carcinoma; BCL, B-cell lymphoma; SCLC, small cell lung cancer. Top plot, cell line count; middle plot, distribution of maximum drug effect on cell viability (E_{max}); bottom plot, median IC_{50} (natural logarithm of μmol/L values). Effects on cell viability were measured by resazurin assay. B, Relative sensitivity of cancer subtypes and statistical enrichment for sensitive cell lines. The volcano plot reports sensitivity to AM-8621 versus statistical significance [-log P value of the Fisher exact test performed using a 10 μmol/L threshold (maximum tolerated dose)] for classification into sensitive and resistant cell lines. Among all individual cancer subtypes included in viability screen, those that demonstrated statistically significant enrichments are shown. T-ALL, T-cell acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia. C, Effects of AM-8621 treatment on the viability of expanded panels of MM (n = 19), AML (n = 28), and DLBCL (n = 21) cell lines. Effects on cell viability were measured by CellTiter-Glo. Mean values from n = 2 to 5 biological replicates for individual cell lines. Black bars report mean ± SD across indication. D, Comparison of sensitivity to AM-8621 versus venetoclax across MM, AML, and DLBCL cell line panels. E, Relationship between BCL-xL RNA expression and sensitivity to AM-8621 for those MM cell lines (n = 14) reported in Fig. 4C and D for which BCL-xL FPKM values were available from the Cancer Cell Line Encyclopedia [42].

AMG 176 and AM-8621 Exhibit Activity in Combination with Clinically Relevant Agents That Target Hematologic Malignancies

Improved long-term survival in multiple myeloma has been achieved through combination therapy with proteasome inhibitors (PI), immunomodulatory drugs, and corticosteroids (e.g., dexamethasone; ref. 28). To investigate the opportunity for combining an MCL1 inhibitor with corticosteroids, we evaluated the effect of AM-8621 combined with dexamethasone in a panel of multiple myeloma cell lines (OPM-2, KMS-11, and MM.1S; ref. 29). AM-8621 further potentiated the cytotoxic effects observed with dexamethasone alone, exhibiting a synergistic interaction in each line (Supplementary Fig. S10). We also evaluated the synergistic potential of AM-8621 combined with the PI carfilzomib. Although a limited synergistic interaction was detected in U266B1 cells, the profound single-agent activity of AM-8621 and carfilzomib in other tested cell lines limited the opportunity...
Figure 5. AMG 176 exhibits robust single-agent activity in vivo. A, Time- and dose-dependent effects of AMG 176 treatment on apoptotic markers (activated BAK, cleaved caspase-3, and cleaved PARP) in established OPM-2 luc tumors as measured in MSD immunoassays. B, Effects of AMG 176 on established OPM-2 luc tumor xenografts when dosed daily or on a 2-day-on, 5-day-off schedule. C, Effects of AMG 176 on established OPM-2 luc tumor xenografts when dosed once weekly. D, Effects of AMG 176 on MOLM13 luc orthotopic tumor xenografts when dosed on a 2-day-on, 5-day-off schedule. Representative day 15 bioluminescence images are shown. For xenograft studies, mean tumor volume or whole-body luminescence (dorsal + ventral image) ± SEM (n = 10/group) are reported. ***P < 0.0001 (RMANOVA with Dunnett post hoc). E, Flow cytometry assessment of B cells, monocytes, and neutrophils in peripheral blood and bone marrow of human MCL1 knock-in mice following administration of AMG 176 24 hours after cycle 1 (day 3) and cycle 2 (day 10). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (one-way ANOVA with Dunnett post hoc).
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for detecting synergy (Supplementary Fig. S11). To further elucidate the therapeutic potential of this combination, we tested AMG 176 and carfilzomib in an orthotopic OPM-2 luciferase-labeled model. In mice treated once-daily with AMG 176 (20 mg/kg) and twice-weekly with carfilzomib (3 mg/kg), the combination achieved significant inhibition of tumor burden (99% reduction in BLI), exceeding the effect achieved with either single agent (85% and 82% reduction in BLI with AMG 176 and carfilzomib, respectively; Fig. 6A).

Combination therapy is a mainstay of AML treatment regimens as well, with induction therapy including nucleoside analogues (e.g., cytarabine), hypomethylating agents (e.g., decitabine), and anthracyclines (e.g., doxorubicin; ref. 30). Our observation that AML cell lines exhibit a range of sensitivities to AM-8621 (Fig. 4C) suggested that combined therapy with standard-of-care (SOC) agents may further sensitize AML tumors to treatment with an MCL1 inhibitor. Thus, we characterized the effects of AM-8621 combined with cytarabine, decitabine, and doxorubicin on four AML cell lines (EOL-1, GDM-1, MOLM13, and MV-4-11). A synergistic interaction was observed with all three combinations across the cell line panel (Fig. 6B; Supplementary Fig. S12), highlighting the potential for combining MCL1 inhibitors with SOC agents in AML.

Cell line profiling studies with AM-8621 and venetoclax demonstrated sensitivity to either MCL1 inhibition alone, BCL2 inhibition alone, or both MCL1 and BCL2 inhibition in many AML lines (Fig. 4D), suggesting that combination therapy may provide benefit beyond selective inhibition of either protein alone. To test this hypothesis, we profiled the same panel of four AML cell lines with the combination of AM-8621 and venetoclax. A synergistic interaction was detected in each cell line (Fig. 6B; Supplementary Fig. S12), highlighting their codependence on MCL1 and BCL2. Next, we tested the combination of AMG 176 and venetoclax in the MOLM13 orthotopic model. Single-agent dose-finding studies revealed significant activity with both compounds (Fig. 5D; Supplementary Fig. S13) and informed dose selection for use in combination. Mice harboring MOLM13 tumors were treated twice-weekly with AMG 176 (30 mg/kg) and daily with venetoclax (50 mg/kg). Whereas both single agents achieved significant reductions in tumor burden (55% and 23% reduction in BLI, respectively; Fig. 6C), the combination exhibited complete inhibition of tumor burden (100% reduction in BLI) and achieved regression relative to the first day of dosing.

We next sought to characterize the effects of this combination on subsets of hematopoietic cells in the human MCL1 knock-in mouse. The treatment schedule and dose for AMG 176 (twice-weekly at 30 mg/kg) and venetoclax (daily at 50 mg/kg) were selected based on observed efficacy in the MOLM13 model. Terminal analysis (24 hours after cycle 2 or day 10) of mice treated with the combination or AMG 176 alone showed significant decreases in peripheral blood B cells and monocytes, whereas venetoclax alone exhibited significant reductions in B cells only (Fig. 6D). The combination was well tolerated, and no evidence of overt toxicity was observed as determined by changes in body weight at the doses selected for this study (Fig. 6E).

AM-8621 was next tested in a panel of primary AML patient samples (Supplementary Table S4). Most of the samples exhibited some degree of sensitivity to AM-8621 treatment, whereas a subset (AML 9, AML 10, and AML 13) were profoundly sensitive (LC50 ≤ 2 nmol/L; Fig. 7A and B). Given the synergy observed with AM-8621 when combined with venetoclax and SOC chemotherapeutics in AML cell lines, we evaluated these combinations in the primary patient samples. Strikingly, in 9 of 13 samples, combination of equimolar concentrations of AM-8621 and venetoclax achieved marked improvements in activity and potency over either single agent alone, whereas only one sample was relatively insensitive to the combination (AML 11; Fig. 7A and B). Combination with idarubicin also exhibited improvement in potency in 6 of 10 samples tested, with 1 sample (AML 2) exhibiting a >1,000-fold improvement. It is important to note that these experiments were conducted in the absence of stromal cells and factors, which are thought to provide a supportive environment for leukemic blasts. Consequently, it should be noted that the absence of these factors may further sensitize the cells to treatment.

**DISCUSSION**

MCL1 is a compelling therapeutic target in cancer. Studies using genetic knockdown and pharmacologic inhibition have demonstrated MCL1’s role as a critical prosurvival factor in many tumor types, including multiple myeloma, AML, B-cell lymphomas, and breast cancer (9, 11–14, 31, 32). Furthermore, MCL1 has been implicated as a resistance factor to BH3 mimetics targeting BCL2 and BCL-xL (33), as well as chemotherapeutic agents (34), further underscoring the therapeutic potential of inhibitors targeting this prosurvival BCL2 family member.

We describe the discovery of AMG 176, a first-in-class MCL1 inhibitor in clinical development for hematologic malignancies. The development of potent and selective MCL1 inhibitors has been challenging due to the high affinity of its native ligands and shallow binding pocket. The discovery of AMG 176 represents an innovative approach for overcoming these significant obstacles. Driving potency, selectivity, and pharmacokinetic properties through conformational restriction guided by structure-based design has yielded a series of rigid macrocyclic inhibitors that primarily displayed the bioactive conformation. The identification of AMG 176 illustrates that conformational restriction provides an effective approach to small-molecule design with potential application for targets previously considered undruggable (17).

Tumor cell line profiling studies with the tool compound, AM-8621, implicated a key role for MCL1 in cell lines derived from hematologic malignancies, including multiple myeloma, AML, and B-cell lymphoma. Whereas AML and DLBCL lines frequently exhibited codependence on MCL1 and BCL2, multiple myeloma lines showed a predominant dependency on MCL1, highlighting the promise for MCL1 inhibitors in this setting. Consistent with prior reports (16, 35), elevated BCL-xL expression was identified as the strongest predictor of resistance. In addition, the expression of BAK was found to be the strongest predictor of sensitivity. These findings are not unexpected considering the redundant prosurvival role of BCL-xL and the function of BAK as the key executioner protein for MCL1. Given the feasibility of measuring these endpoints in the clinic, both BCL-xL and BAK may serve as biomarkers for patient stratification and shed light on potential mechanisms of resistance to MCL1 inhibitors in the clinic.
Figure 6. AMG 176 exhibits activity when combined with clinically relevant agents that target hematologic malignancies. A, Combination effects of AMG 176 plus carfilzomib on OPM-2 luc orthotopic tumor xenografts. Mean hind limb bioluminescence ± SEM (n = 10/group) is reported for each group. 

B, Synergy scores for AMG 176 and SOC chemotherapeutics (cytarabine, decitabine, and doxorubicin) or venetoclax. Effects on cell viability were measured by CellTiter-Glo. Higher scores reflect stronger synergistic interaction (darker red). C, Combination effects of AMG 176 plus venetoclax on MOLM13 luc orthotopic tumor xenografts. Mean whole-body bioluminescence (dorsal + ventral image) ± SEM (n = 10/group) is reported for each group. 

D, Flow cytometry assessment of B cells, monocytes, and neutrophils in peripheral blood from human MCL1 knock-in mice treated with AMG 176 alone or combined with venetoclax 24 hours after cycles 1 (day 3) and 2 (day 10). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (one-way ANOVA with Dunnett post hoc). E, Observed body weights in human MCL1 knock-in mice following treatment with AMG 176, venetoclax, or AMG 176 and venetoclax combined. Data are reported as % mean average body weight compared with % pretreatment body weight ± SEM (n = 5/group).
AMG 176 Alone and Combined in Hematologic Cancer Models

Figure 7. AMG 176 is active against primary AML samples as a single agent and when combined with clinically relevant therapeutics. A, Dose–response curves showing primary AML samples gated on blasts and viability assessed after 48-hour incubation with indicated drugs. B, Normalized LC\textsubscript{50}(50% lethal concentration) viability values comparing AM-8621 combined with indicated drugs after 48 hours of treatment.

A notable feature of this class of MCL1 inhibitors is their rapid induction of apoptosis in hematologic cancer cell lines, providing clear rationale for testing the clinical molecule AMG 176 with discontinuous dosing strategies. In vivo, robust TGI was observed with once- and twice-weekly dosing schedules. These findings have important implications for the clinical development of AMG 176, as they offer the promise of greater flexibility in dosing schedules to mitigate potential on-target toxicities without sacrificing efficacy.

Given the lack of activity of AMG 176 on murine MCL1, we generated a human MCL1 knock-in mouse to facilitate an improved understanding of the relationship between MCL1 inhibition at efficacious exposures and its effects on normal tissues. In contrast to published MCL1 gene ablation studies where MCL1 knockout resulted in lethality, inhibition of MCL1 at efficacious and pharmacodynamically active doses of AMG 176 was tolerated in the knock-in mice (36, 37). In addition, for the first time we have demonstrated an effect on normal tissues with an MCL1 inhibitor at tolerated and efficacious exposures. Consistent with data from conditional MCL1 gene ablation studies, AMG 176 treatment significantly reduced B cells, monocytes, and neutrophils at exposures required for TGI (24–27). These data suggest that reductions in these cell types may serve as pharmacodynamic endpoints.

The codependency of many cell lines on MCL1 and BCL2 suggests that their combined inhibition has the potential for improved efficacy in indications such as AML. This hypothesis was supported by the robust activity observed with the combinations of AM-8621/AMG 176 and venetoclax in AML cell lines, xenograft models, and primary patient samples. We also utilized human MCL1 knock-in mice to test the relationship between the tolerability of this combination and effects on
pharmacodynamic endpoints. Administration of venetoclax and AMG 176 on a schedule achieving continuous BCL2 and intermittent MCL1 inhibition was well tolerated while demonstrating significant reduction in B cells and monocytes. Given this compelling data, combined treatment with AMG 176 and venetoclax has promising therapeutic potential in AML.

The dependency of a broad range of hematopoietic tumor types on MCL1 for survival highlights the exciting therapeutic promise for MCL1 inhibitors. AMG 176 is the first MCL1 inhibitor to enter clinical development and has the potential to significantly expand the clinical opportunity for BH3 mimetics in the treatment of cancer.

**METHODS**

**Clinical Candidate Synthesis**

AMG 176 (15,3′R,6′R,7′R,8′E,11′S,12′R)-6-chloro-7′-methoxy-11′,12′-dimethyl-3,4-dihydro-2H,15′H-spiro[naphthalene-1,2′-][20]oxa[13]thia[1,4]diazatetraacyclo[4.7.2.03.6.019.24]pentacosa[8,16,18,24]tetraen]-15′-one-13′, 13′-dioxide) and other small-molecule inhibitors of MCL1 described were synthesized at Amgen Inc. (38).

**Conformational Energy Calculations**

Details of conformational energy calculations are described in the Supplementary Information.

**Time-Resolved Fluorescence Resonance Energy Transfer Binding Assays**

Recombinant 6His-tagged human MCL1 (171-327), dog MCL1 (171-327, C286S), mouse MCL1 (152-308), and BCL-xL (1-196) were produced at Amgen Inc. Proteins were expressed in Escherichia coli (E. coli) and purified using metal ion affinity chromatography and size-exclusion chromatography. Recombinant 10His-tagged human BCL2 (2-211) was purchased from R&D Systems. Human Biotin-Bim BH3 peptide (Biotin-DLRPEIRIAQELRRIGDENVAYRR) and mouse Biotin-Bim BH3 peptide (Biotin-DLRPEIRIAQELRRIGDEFNETYTR) were custom-synthesized by CPC Scientific.

Inhibition of the interaction between Biotin-Bim BH3 peptide and MCL1, BCL2, or BCL-xL was measured using time-resolved fluorescence resonance energy transfer (TR-FRET) assays conducted in 384-well white OptiPlates (PerkinElmer) with a total volume of 40 μL well in binding buffer (20 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 0.016 mmol/L Brij35, and 1 mmol/L DTT). Serially diluted test compounds were preincubated with Biotin-Bim BH3 peptide and protein (MCL1, BCL2, or BCL-xL) for 60 minutes before addition of the detection mixture [LANCE Eu-W1024 Anti-60His (PerkinElmer) and Streptavidin-XLent (Cisbio US)]. Plates were further incubated overnight and then read on an EnVision Multilabel Reader (PerkinElmer; see Supplementary Information).

**Fluorescence Polarization Binding Assay**

A fluorescence polarization (FP) assay was used to measure inhibition of the human MCL1/BIM interaction. Human MCL1 (171-327) was prepared as described above. TAMRA-labeled BIM BH3 peptide (TAMRA-GWIAQELRIRGDEFG) was produced internally using standard Fmoc chemistry. The FP binding assay was conducted in 384-well black OptiPlates with a total volume of 40 μL well in the same binding buffer used in the TR-FRET assay. Serially diluted test compounds were incubated with 13.2 mmol/L human MCL1 and 5 mmol/L TAMRA-labeled BIM BH3 peptide for 120 minutes before FP was measured on an EnVision Multilabel Reader with excitation and emission filters at 531 and 595 nm, respectively, and a dual 555/595 nm dichroic mirror. IC50 values were determined as described in the TR-FRET methods (see Supplementary Information).

**MCL1 Crystallography and Structure Determination**

Human MCL1 (171-327) with a cleavable N-terminal GST tag was expressed in *E. coli* and purified using glutathione affinity chromatography. The N-terminal GST tag was cleaved by thrombin. Untagged MCL1 was purified by cation exchange chromatography. Purified MCL1 (10 mg/mL) was incubated with 3-fold excess amount of inhibitor at 4°C for 30 minutes before crystalization. Crystals of MCL1 with inhibitors were obtained at 4°C in hanging drops with 100 mmol/L Tris, pH 8.0, 3% methanol, and 36% to 42.5% PEG6000. Paratone-N mineral oil was used as cryoprotectant. Diffraction data were collected on beamline 21-ID-F at the Advanced Photon Source and processed and scaled with HKL 2000. Thecocystal structures were solved by molecular replacement with AMoRe using PDB entry code 2PQK as the template. Model building was performed with COOT with REFMAC refinement.

**NMR and Isothermal Titration Calimetry**

Due to the limited solubility of compound 1, the isothermal titration calorimetry (ITC) with the VP-ITC instrument (Malvern, Inc.) was performed in the “reverse” mode, where the protein (100 μmol/L) was titrated into the low concentration (7 μmol/L) of the ligand. NMR experiments were performed using a Bruker 800 MHz spectrometer equipped with the TCI cryoprobe and U-15C,13N-hMCL1 C893S protein (39–41). Spectral assignments of key residues in the hMCL1:2 complex were based on the standard nuclear Overhauser effect (NOE) spectroscopy (NOESY) with heteronuclear single quantum coherence spectroscopy (NOESY-HSQC) experiment, performed on a sample prepared as a 1:1 mixture of hMCL1:2 complex and apo-hMCL1, where the letter was previously assigned. This experiment was performed at 310K to enhance chemical-exchange rates (up to 2 s−1) between free and bound protein forms. The intraligand and ligand to protein NOEs were obtained from the 2-D X-filtered NOESY and 3-D X-filtered NOESY-HSQC as described previously (41). The 3-D structure of the hMCL1:2 complex was obtained by the “NOE guided” docking of 2 to the whole NMR ensemble (20 structures) of the apo-hMCL1. Computations were performed in-house–developed scripts in Matlab; the final structure was energy-minimized in Molecular Operating Environment software.

**Cell Lines**

Tumor cell lines were obtained from commercially available sources including the American Type Culture Collection (ATCC), Japanese Collection of Research Bioresources (JCRB), and German Collection of Microorganisms and Cell Cultures (DSMZ). Cell lines were passaged for <1 month before banking and experimentation. With the exception of the 952 cell line profiling screen, all cell lines were cultured in ATCC-, DSMZ-, or JCRB-recommended growth media containing 10% FBS, except where specified. Growth media and culture conditions for the 952 cell line profiling screen were described previously (23). Authentication of cell lines was performed with short tandem repeat DNA typing. Using an RT-PCR–based assay, all cell lines used for *in vitro* studies were tested for *Mycoplasma* contamination before use; cell lines used in *in vivo* studies were tested periodically.

**Split Luciferase Complementation Assay**

HEK293M cells were transiently transfected with pcDNA mammalian expression vectors encoding amino acids 1–298 of firefly luciferase fused to human BAK [pcDNA-Luc (1–298)–BAK] and amino acids 395–550 of firefly luciferase fused to human MCL1 [pcDNA-Luc (395–550)–MCL1] at a ratio of 3:1, respectively. Transient transfection was performed using Lipofectamine LTX and PLUS reagent (ThermoFisher). Twenty-four hours after transfection, cells were
collected using non–enzyme-based cell dissociation buffer Accutase (Innovative Cell Technologies) and resuspended in Opti-MEM (ThermoFisher) without serum. Cells were seeded in 96-well assay plates (5,000 cells/well) and treated with AM-8621 for 4 hours. Following compound treatment, 30 μL of Steady-Glo Luciferase detection reagent (Promega) was added to each well. Signal was read on an EnVision Multilabel Reader. Peak luciferase signal in DMSO-treated wells was normalized to percent of control (POC) = 100. Luciferase signal from no cell control wells was normalized to POC = 0.

**Immunoblot Analysis**

Details of the immunoblot experiments are described in the Supplementary Information.

**qPCR Analysis of MCL1 Transcript**

U266B1 cells were seeded at a density of 3 × 10^6 cells/cm² tissue culture dish and incubated overnight at 37°C in 5% CO₂. Cells were treated with indicated concentrations of AM-8621 for 24 hours followed by wash with PBS. RNaseasy Mini Kits (Qiagen) were used to isolate RNA from cells per the manufacturer’s protocol. MCL1 transcript levels were measured with the TaqMan One-Step RT-PCR Master Mix Reagents Kit and TaqMan Gene Expression Assays (ThermoFisher). Quantitative qRT-PCR were run as four technical replicates and assayed using the Prism 7900HT (Applied Biosystems), applying the relative quantification (ΔΔCt) method. Data were analyzed with SDS2.3, RQ Manager, and Data Assist v3.01 software (Applied Biosystems), using glyceraldehyde 3-phosphate dehydrogenase as the endogenous control.

**MCL1 Half-Life Experiments**

U266B1 cells were seeded at a density of 1.5 × 10^6 cells/well in 6-well tissue culture plates and incubated overnight at 37°C and 5% CO₂. Cells were pretreated with DMSO or AM-8621 at a concentration of 2 μmol/L for 4 hours. Cycloheximide was then added at a final concentration of 100 μg/mL to arrest bulk translation. Cell lysates were harvested at indicated time points after cycloheximide addition and subjected to immunoblot analysis.

**Caspase 3/7 Activity Assay**

Cells were seeded at optimized densities in 96-well tissue culture plates, incubated overnight at 37°C in 5% CO₂, and treated with AM-8621 at indicated concentrations for 0.5, 1, 2, 4, 8, or 24 hours. Plates were equilibrated to room temperature (RT) for 30 minutes before the addition of reconstituted Caspase-Glo 3/7 reagent (Promega). Plates were shaken for 2 minutes at RT followed by incubation for 30 additional minutes without shaking. Luminescence was read on an EnVision Multilabel Reader; 100% caspase 3/7 activity was defined as the maximal observed signal for a given cell line over the course of the experiment.

**AM-8621 Washout Viability Studies**

Cells were seeded at optimized densities in 96-well tissue culture plates, incubated overnight at 37°C in 5% CO₂, and treated with a 9-point serial dilution of AM-8621, using a top concentration of 6.7 μmol/L, 1:3 serial dilution steps, and a DMSO-only control. Following compound treatment for indicated durations, cells were washed 4 times and returned to growth media in the absence of compound for the duration of the 24-hour experiment. Effects on cell viability were measured with the CellTiter-Glo viability assay (Promega) as follows. Treated cells and CellTiter-Glo Luminescent Cell Viability Assay reagents (Promega) were allowed to equilibrate to RT, and 100 μL aliquots of reconstituted CellTiter-Glo reagent were added to each well of AMG-8621-treated cells. Assay plates were shaken for 2 minutes followed by incubation at RT for 10 minutes. Plates were then read on an EnVision Multilabel Reader.

**Tumor Cell Line Profiling Screens**

Details of the tumor cell line profiling experiments are described in the Supplementary Information.

**Elastic Net Model**

Details of the elastic net model are described in the Supplementary Information.

**BCL-xL Immunoassay**

Lysates from indicated multiple myeloma cell lines were prepared in MSD lysis buffer. Custom BCL-xL capture plates were developed using a total BCL-xL antibody (R&D Systems, 840767). Capture plates were blocked in MSD blocking solution A for 1 hour at RT with shaking. Plates were then washed 3 times, followed by the addition of 20 μg/well of 1:10 BCL-xL detection antibody (Cell Signaling Technologies, 2746). Plates were incubated at RT for 1 hour with shaking. Plates were then washed 3 times followed by the addition of MSD read buffer. Plates were read on an MSD S16000 plate reader. To obtain BCL-xL protein levels for individual multiple myeloma cell lines, a standard curve of AGS (a multiple myeloma cell line with high BCL-xL expression) lysate was run on each plate to ensure lysates from individual multiple myeloma cell lines fell within the linear range of the assay. Relative BCL-xL protein levels were then calculated using the AGS standard curve.

**MCL1:BIM Complex Immunoassay**

Details of the immunoassay are reported in the Supplementary Information.

**In Vitro Combination Studies**

*In vitro* combination studies were carried out as previously described (29); additional details are described in the Supplementary Information.

**Generation of Human MCL1 Knock-In Mouse**

Human MCL1 knock-in mice were created by targeting C57BL/6 embryonic stem cells (The Jackson Laboratory) with a targeting vector containing the full human MCL1 genomic locus flanked by homologous mouse sequences upstream and downstream of the mouse MCL1 genomic locus. Additional detail is included in the Supplementary Information.

**Ex Vivo Analysis of Mouse Splenocytes**

Details are described in the Supplementary Information.

**Flow Cytometry-Based BAK Activation Assay**

Details are described in the Supplementary Information.

**Pharmacodynamic Evaluation of Active BAK, Cleaved PARP, and Cleaved Caspase-3 in Subcutaneous Human Tumor Models**

Pharmacodynamic methods are described in the Supplementary Information.

**Immunohistochemistry**

Immunohistochemistry experiments were performed as described in the Supplementary Information.

**Subcutaneous Human Tumor Models**

Multiple myeloma cells (cell line OPM-2 luc) were injected s.c. in the right flank of mice (5 × 10⁶ cells). Tumor volume (mm³) was...
measured using electronic calipers twice per week. Once tumors reached an average of approximately 150 mm³, animals were randomized into groups (n = 10 per group) such that the average tumor volume at the beginning of treatment administration was uniform across treatment groups. Animals were then orally administered with AMG 176 daily, 2×/week, or 1×/week. Clinical signs, body-weight changes, and tumor growth were measured 2×/week until study termination.

**Orthotopic Human Tumor Models**

Firefly luciferase-labeled OPM-2 or MOLM13 cells were injected i.v. into the tail vein of NOD/SCID IL2rg or athymic nude mice, respectively. Tumor BLI was measured using Xenogen IVIS 200 twice per week. Once tumors reached an average BLI of 1 × 10³ photons/second, animals were randomized into groups (n = 10 per group) such that the average BLI at the beginning of treatment administration was uniform across treatment groups. Animals were then orally administered AMG 176 daily for carfilzomib combination studies and 2×/week for venetoclax combination studies. Carfilzomib was administered i.v. 2×/week for 6 doses, beginning on the same day at the same time as AMG 176. Venetoclax was administered orally every day, beginning on the same day, 4 hours after AMG 176 administration. Clinical signs, body-weight changes, and tumor BLI were measured 2 times per week until study termination.

**Flow Cytometry Analysis of Monocytes and B Cells from Human MCL1 Knock-In Mice**

Details are described in the Supplementary Information.

**CBC Analysis**

Details described in the Supplementary Information.

**Statistical Analysis of In Vivo Studies**

Single-agent *in vivo* efficacy data were analyzed by RMANOVA followed by Dunnett correction. Combination *in vivo* efficacy data were analyzed by repetitive two-group RMANOVA analyses between the combination group and each of the relevant single-agent controls. One-way ANOVA with Dunnett correction was applied for analysis of flow cytometry data.

**Animal Care**

Cages were changed once per week. Harlan Teklad Sterilizable Rodent Diet 8656 and reverse-osmosis water from the Amgen water supply system were supplied *ad libitum*. Diet 8656 and reverse-osmosis water from the Amgen water supply system were supplied *ad libitum*. Controls were maintained in a temperature-controlled environment with a 12-hour light cycle and met all Association for Assessment and Accreditation of Laboratory Animal Care specifications. All cages provided a 12-hour light cycle and met all Association for Assessment and Accreditation of Laboratory Animal Care specifications. Harlan Teklad Sterilizable Rodent Diet 8656 and reverse-osmosis water from the Amgen water supply system were supplied *ad libitum*. Cows were then orally administered with AMG 176 daily, 2×/week, or 1×/week. Clinical signs, body-weight changes, and tumor growth were measured 2×/week until study termination.

**Primary Patient Samples**

Details described in the Supplementary Information.

**Ex Vivo Drug Testing of Primary AML Patient Samples**

Details described in the Supplementary Information.

**Disclosure of Potential Conflicts of Interest**

S. Caenepeel is Senior Scientist at Amgen Inc. and has ownership interest (including stock, patents, etc.) in Amgen Inc. A.C. Cheng has ownership interest (including stock, patents, etc.) in Amgen Inc. J. Houze has ownership interest (including stock, patents, etc.) in Amgen Inc. B. Lucas has ownership interest (including stock, patents, etc.) in Amgen Inc. N.A. Paras is Associate Professor at UCSF Institute for Neurodegenerative Diseases. A. Coxon has ownership interest (including stock, patents, etc.) in Amgen Inc. P.E. Hughes has ownership interest (including stock, patents, etc.) in Amgen Inc. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


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**Other (contributed to design of AMG 176 and its synthesis):** N.A. Paras

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REFERENCES

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