

AMG 925 Is a Dual FLT3/CDK4 Inhibitor with the Potential to Overcome FLT3 Inhibitor Resistance in Acute Myeloid Leukemia

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Abstract

Resistance to FLT3 inhibitors is a serious clinical issue in treating acute myelogenous leukemia (AML). AMG 925, a dual FLT3/CDK4 inhibitor, has been developed to overcome this resistance. It is hypothesized that the combined inhibition of FLT3 and CDK4 may reduce occurrence of the FLT3 resistance mutations, and thereby prolong clinical responses. To test this hypothesis, we attempted to isolate AML cell clones resistant to AMG 925 or to FLT3 inhibitors. After a selection of over 8 months with AMG 925, we could only isolate partially resistant clones. No new mutations in FLT3 were found, but a 2- to 3-fold increase in total FLT3 protein was detected and believed to contribute to the partial resistance. In contrast, selection with the FLT3 inhibitors sorafenib or AC220 (Quizartinib), led to a

resistance and the appearance of a number of mutations in FLT3 kinase domains, including the known hot spot sites D835 and F691. However, when AC220 was combined with the CDK4 inhibitor PD0332991 (palbociclib) at 0.1 $\mu\text{mol/L}$ or higher, no resistance mutations were obtained, indicating that the CDK4-inhibiting activity of AMG 925 contributed to the failure to develop drug resistance. AMG 925 was shown to potentially inhibit the FLT3 inhibitor-resistant mutation D835Y/V. This feature of AMG 925 was also considered to contribute to the lack of resistance mutations to the compound. Together, our data suggest that AMG 925 has the potential to reduce resistance mutations in FLT3 and may prolong clinical responses. *Mol Cancer Ther*; 14(2): 375–83. ©2014 AACR.

Introduction

Approximately 25% of patients with acute myelogenous leukemia (AML) have internal tandem duplication (ITD) mutations in the FLT3 gene (Fms-like tyrosine kinase 3, FLT3-ITD), which cause constitutive activation of the kinase and dysregulated cell proliferation (1–3). Patients with FLT3-ITD mutations in their tumors have poor prognoses, emphasizing the importance of FLT3 in the progression of the disease (1, 3, 4). Although FLT3 has been pursued as a drug target for treating AML, the development of FLT3 inhibitors has met with serious challenges. The major issue has been the development of drug resistance within only a few months after the initial responses to treatment (5, 6). Some of the earlier FLT3 inhibitors are nonselective multikinase inhibitors (7–9). A lack of sufficient target coverage due to dose-limiting toxicities is thought to be a possible reason for quick development of drug resistance (7–9). Sorafenib is a more selective FLT3 inhibitor than some other previous FLT3 inhibitors, yet, it also led to only transient clinical responses (10, 11). AC220

(quizartinib), the most potent and selective FLT3 inhibitor reported so far (12, 13), has entered clinical trials (14). Maximal target coverage could be achieved with doses below the maximally tolerated dose (14). AC220 demonstrated a high complete remission rate (54%) in treating FLT3-ITD (+) AML (14). However, the median clinical response yet still lasted about only 3 months (14). More intriguingly, newly acquired secondary mutations at D835 and F691 in the FLT3 kinase domains were found in all of the relapsed patients (15). These mutations were demonstrated to confer resistance to AC220 *in vitro*. It is now clear that FLT3 inhibition alone is unlikely to lead to a durable clinical response in treating AML due to the genome instability intrinsic to AML cells and the ease with which they acquire FLT3 resistance mutations (16, 17).

AMG 925 is a novel potent and selective FLT3/CDK4 dual-kinase inhibitor designed to address the issue of resistance to FLT3 inhibitors (18, 19). CDK4 is a Cyclin D-dependent kinase that plays an essential role in integrating external growth signals and promoting progression from G₁ to S phase of the mammalian cell cycle (20, 21). CDK4 functions to phosphorylate and inactivate the Rb protein. The inactivated Rb then releases E2F transcription factors to initiate S phase of the cell cycle (22, 23). The role of the CDK4–Rb pathway in cancer is supported by strong genetic and pharmacologic evidence (24–27). Rb, the key substrate of CDK4, and p16, a member of the INK4 family of cellular inhibitor of CDK4, are both well-defined tumor suppressors (28, 29). The genes encoding the two tumor-suppressor proteins are often deleted in various cancers (24). The selective CDK4 inhibitor PD0332991 (palbociclib) has recently shown impressive anticancer efficacy in treating estrogen-dependent advanced

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breast cancer in combination with Letrozole, an estrogen synthesis inhibitor (27). Although deletion of the p16 gene is uncommon in AML, epigenetic downregulation of p15, another INK4 family protein, was reported in up to 60% of patients with AML (30, 31). In AML cell lines carrying FLT3-ITD mutations, expression of CyclinD2 and D3, two partnering cyclins of CDK4, is highly upregulated (32). In addition, CDK4 inhibition has been shown to enhance the apoptotic effects of FLT3 inhibitors in these AML cell lines (32). These observations suggest that CDK4 is also an attractive drug target for AML. We hypothesize that the combined inhibition of FLT3 and CDK4 by the dual inhibitor AMG 925 may provide more durable responses than the sole inhibition of FLT3 by FLT3 inhibitors such as AC220 and sorafenib. In this study, we demonstrated that AML cell lines did not develop complete resistance to AMG 925, and the observed partial resistance was not due to FLT3 mutations, in contrast with the case with FLT3 inhibitors sorafenib and AC220. The mechanism for the failure to develop full resistance to AMG 925 is suggested by the failure of FLT3 mutations to appear when AC220 is combined with the CDK4 inhibitor PD0332991. In addition, panels of FLT3 inhibitor resistance mutants were used to test sensitivities to AMG 925, and confirmed that AMG 925 potently inhibited FLT3 D835-mutant activity. This feature of AMG 925 also likely contributed to the inability to isolate resistant clones to AMG 925 in AML cell lines.

Materials and Methods

Compounds

AMG 925 was synthesized at Amgen. PD0332991, AC220, and sorafenib were purchased from AdooQ BioScience.

Cell lines and proliferation assay

MOLM13 and MV11-4 were obtained from the DSMZ German Collection of Microorganisms and Cell Cultures (Germany). U937, MDA-MB-436, 293T, and Ba/F3 were obtained from the ATCC. All cell lines were grown in RPMI-1640 medium supplemented with 10% FBS, 50 U/mL penicillin and streptomycin. For Ba/F3 cells, 2 ng/mL of IL3 (R&D Systems) was added to the medium. All cell lines used in this study were authenticated by short tandem repeat DNA profiling at Genetica DNA Laboratories.

For proliferation assays in 96-well plates, 6,000 cells per well were seeded and treated the same day with serially diluted compounds for 3 days. Cell viability was measured using the CellTiter-Glo assay (Promega). Dose-response curves were plotted to determine IC_{50} values for the compounds using GraphPad Prism v5.01 software (GraphPad Software).

Apoptosis

Apoptosis of the AML cells treated with compounds was determined by using a Vybrant Apoptosis Assay Kit following the manufacturer's protocol (Invitrogen). Briefly, cells in exponential growth phase were seeded into a 6-well plate at 5×10^5 cells per well and treated with compounds at indicated concentrations for 24 hours. Cells were then stained followed by flow cytometry analysis. FlowJo software (eBioscience, Inc.) was used to quantify live, apoptotic, and dead cells in the assay.

Generation of FLT3 inhibitor-resistant AML cell clones

MOLM13 and MV4-11 cells were cultured in medium containing AMG 925, sorafenib, or AC220. All compounds were used at

0.001 μ mol/L initially and the concentration was doubled when cell viability reached 90%.

To determine the effect of CDK4 inhibition on the occurrence of resistance to FLT3 inhibitor AC220, MV4-11 cells were cultured in medium containing AC220, in the presence or absence of CDK4 inhibitor PD0332991. Concentration of PD0332991 was fixed at 0.001, 0.01, 0.1, and 1 μ mol/L while gradually increasing AC220 from 0.001 μ mol/L.

Ten independent isolations were performed for each treatment. Total RNA was isolated from each clone and reverse transcription was performed to generate cDNA. The primers TK1F (5'-TGCTGTGCATACAATCCCTTGGC-3') and TK2R (5'-TCTCTGCTGAAAGTTCGCCTGTTT-3') were used for FLT3 kinase domain amplification. Subsequent bidirectional sequencing was performed using these primers in addition to TK1R (5'-AGTCCTCCTCTCTTCCAGCCTTT-3') and TK2F (5'-GAGAGGCACTCATGTGCAACTCA-3'). Alignments to native FLT3 sequence were performed using BLAST online.

Generation of Ba/F3 clone expressing various human FLT3 mutants

cDNAs carrying the human FLT3 gene with various mutations, including ITD, ITD/D835Y, ITD/F691I, ITD/Y842N, ITD/N676K, and D835Y, were cloned into pDONR221 vector (Life Technologies) by GenScript. FLT3-ITD was generated by inserting 21 bp of sequences (TTGATTTTCAGAGAATATGAAT) into FLT3 coding sequence region between 1774 and 1775. Variants of FLT3 constructs were then shuttled into pRV406G vector by using the Gateway LR Clonase Enzyme Mix Kit following the manufacturer's protocol (Life Technologies). Subsequently, pRV406G with mutated FLT3 and Ecopackaging plasmids were cotransfected into 293T cells using lipofectamine (Life Technologies) as per the manufacturer's protocol. Viral supernatants were collected at 48 hours.

Stable Ba/F3 lines were generated by using retroviral spinfection with the appropriate mutated plasmids. At 48 hours after infection, G418 was added to infected cells at a concentration of 500 μ g/mL. Cells were selected in the presence of G418 for 10 days, and subsequently IL3 was washed twice from the cells with media and cells were allowed to grow in RPMI-1640 and 10% FBS without IL3.

Generation of FLT3 inhibitor-resistant Ba/F3 FLT3-ITD cell clones

Ba/F3 FLT3-ITD cells were treated with 100 μ g/mL mutagen N-ethyl-N-nitrosourea (ENU; Sigma) for 16 hours in growth medium. Cells were then washed three times with medium and treated with AMG 925 at 0.5 μ mol/L or AC220 at 0.05 μ mol/L. Distribute 40,000 cells per well into 4×96 -well plates for each compound. Check cell viability every 2 to 3 days. In about 4 to 6 weeks, transfer and expand surviving cells to 24- and 6-well plates containing AMG 925 or AC220 of the original concentrations. Genomic DNA was isolated for FLT3 kinase domain sequencing. PCR and sequencing primers are the same as used for sequencing the AML cell clones.

STAT5 phosphorylation

To determine the levels of phospho-STAT5 (P-STAT5), cells were seeded in a 96-well cell culture plate at a density of 20,000 cells per well. Serially diluted compounds were then added to each well. Cells were harvested after 1-hour treatment and P-STAT5

(Tyr694) and total STAT5 were determined and quantified by using a MSD kit (cat# K11163D; Meso Scale Discovery) following the manufacturer's protocols. Total protein (10 μ g) from each sample was analyzed.

Western and immunoprecipitation Western blotting

For Western blotting, 25 μ g total protein/lane were subjected to SDS-PAGE electrophoresis and immunoblotted with antibodies against P-ERK, T-ERK, P-AKT, T-AKT, P-STAT5, T-STAT5, P-RB, T-RB, and β -actin (all from Cell Signaling Technology). Immunoprecipitation Western blotting for T-FLT3 and P-FLT3 was performed as previously described (18).

Results

CDK4 inhibition enhances apoptosis induced by blocking FLT3 signaling in AML lines bearing FLT3-ITD

The effects of combining the CDK4 inhibitor PD0332991 with FLT3 inhibitors sorafenib and AC220 were evaluated by an apoptosis assay. MV4-11 and U937 cells were treated with 0.5 μ M PD0332991 in combination with different concentrations of sorafenib and AC220. PD0332991 enhanced apoptosis induced by both compounds at a range of concentrations (Fig. 1, top). Similar results were observed in another AML cell line MOLM13 (data not shown). No such enhancement was observed in U937 (Fig. 1, bottom), which carries wild-type FLT3 and its growth is independent of FLT3. These data demonstrate that CDK4 inhibition could enhance cell killing of an FLT3 inhibitor when used in combination in AML cells bearing FLT3-ITD.

Isolation and characterization of AMG 925-resistant AML cell clones

As previously reported (18, 19), AMG 925 is a potent and selective dual FLT3/CDK4 inhibitor. It potently inhibits FLT3 and CDK4 kinases and the growth of FLT3-dependent AML cell lines bearing FLT3-ITD, MOLM13, and MV4-11 (Supplementary Table S1). The specific cellular activities of AMG 925 in blocking FLT3

signaling was demonstrated by inhibition of phosphorylation of STAT5, a well-validated pharmacodynamics (PD) marker for the FLT3 pathway (33). Inhibition of CDK4 in cells was demonstrated by growth inhibition of FLT3-independent and Rb-positive (Rb⁺) cell lines (Supplementary Table S1).

AMG 925-resistant AML cell clones MOLM13^{925R} and MV4-11^{925R} were isolated following protocols as described in Materials and Methods. The highest concentration of AMG 925 at which the resistant cells could grow was 0.1 μ M/L after 8 months of selection. No secondary mutations were found by sequencing FLT3 cDNA from these clones (our unpublished data).

MOLM13^{925R} and MV4-11^{925R} behaved similarly in growth inhibition assays. Both were partially resistant to AMG 925 with growth inhibition IC₅₀ values increased by only 3- to 5-fold compared with parental MOLM13 and MV4-11 cells. Results from MV4-11 cells showed in Fig. 2A, left. In addition, cell doubling time was significantly increased, from 48 hours for parental cells to 72 hours. The resistance phenotype was reversible and the cells regained full sensitivity to AMG 925 in a week after compound removal (Fig. 2A, right). Both MOLM13^{925R} and MV4-11^{925R} clones were similarly cross-resistant to AC220 and sorafenib as expected (Supplementary Table S2).

To understand the resistance mechanism, the FLT3 signaling pathway was studied in the cells resistant to AMG 925. We compared phosphorylation of FLT3, STAT5, ERK, and AKT in MV4-11 and MV4-11^{925R} cells in the presence of 0.1 μ M/L AMG 925. Although phosphorylation of FLT3 (P-FLT3), STAT5 (P-STAT5), ERK (P-ERK), and AKT (P-AKT) was inhibited in MV4-11, 2- to 3-fold increases in P-FLT3, P-ERK, and P-STAT5 were observed in MV4-11^{925R} cells (Fig. 2B, left). Unlike STAT5, ERK and AKT, total FLT3 (T-FLT3) in MV4-11^{925R} cells also increased. No increase in FLT3 mRNA levels was detected by quantitative RT-PCR (our unpublished data), suggesting a post-transcriptional mechanism. The MV4-11^{925R} cells were next treated with the protein synthesis inhibitor cycloheximide. After addition of cycloheximide, the cell samples were collected and processed at various time points to determine levels of T-FLT3 by immunoprecipitation Western blotting (Fig. 2B, right). At 3 hours after the addition

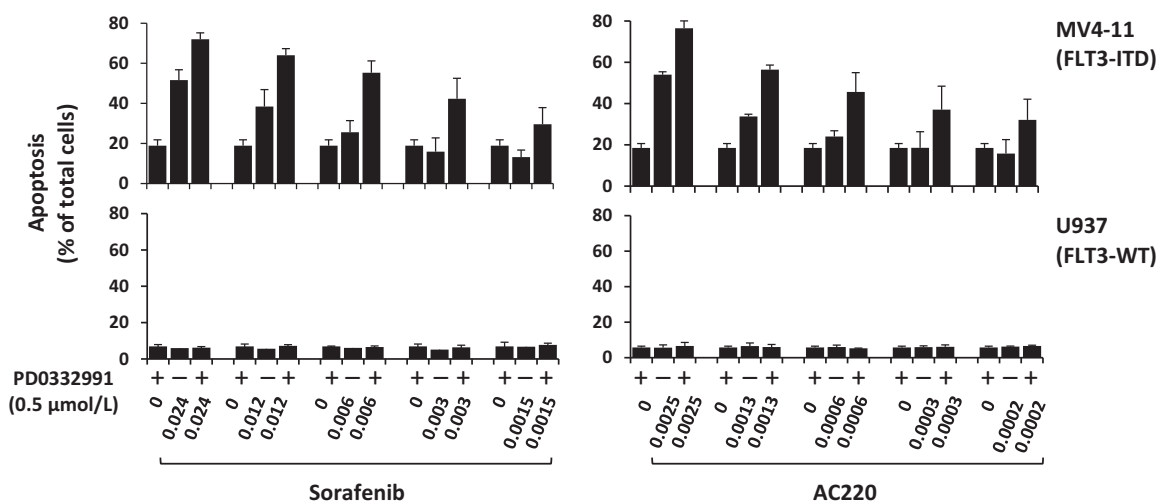
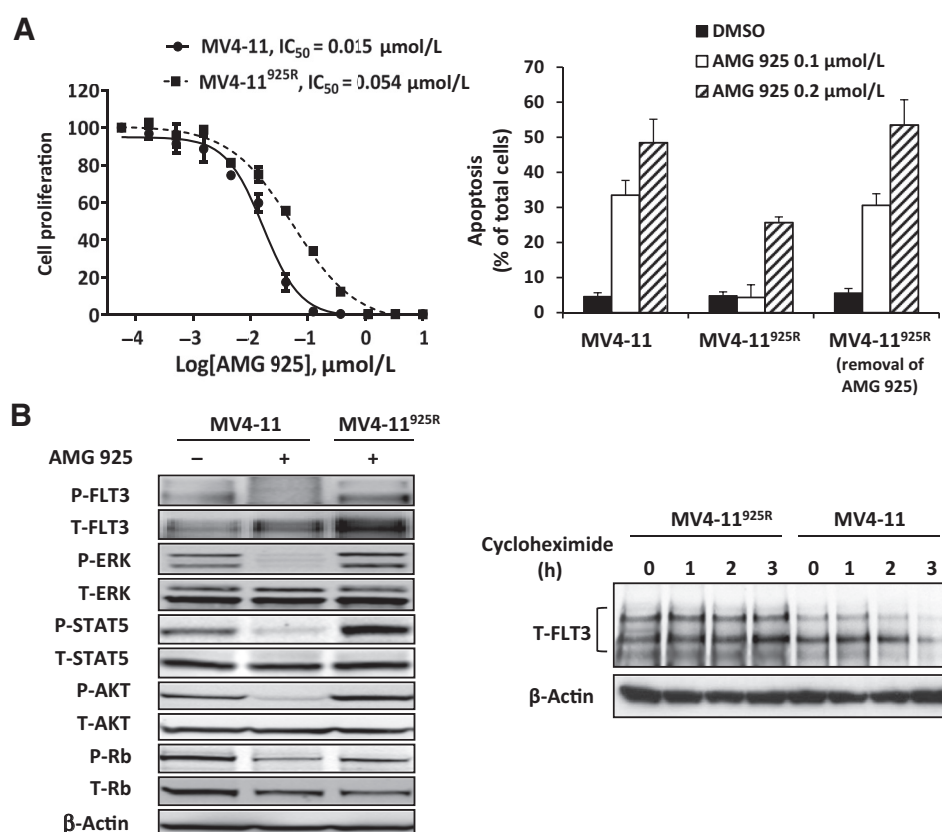


Figure 1.

CDK4 inhibition enhances apoptosis induced by FLT3 inhibitors in AML cells. MV4-11-bearing FLT3-ITD mutation or U937-bearing FLT3-WT were seeded into 6-well cell culture plates before compound treatment. After 24-hour treatment with compounds as indicated, cells were harvested for apoptosis analysis by Annexin V APC/Sytox Green staining. PD0332991 is a selective CDK4/6 inhibitor. AC220 and sorafenib are FLT3 inhibitors. The assays were set up in duplicate plates; error bars, \pm SD.

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**Figure 2.**

Characterization of AMG 925-resistant AML cells. A, MV4-11^{925R} is partially resistant to AMG 925. MV4-11^{925R} cells were obtained by prolonged incubation of parental MV4-11 cells in the presence of increasing concentrations of AMG 925. Left, MV4-11^{925R} cells were partially resistant to AMG 925 as determined by cell growth inhibition assays. Right, MV4-11^{925R} cells were partially resistant to AMG 925 and the resistance was reversible as determined by apoptosis assays. Growth inhibition was determined by a CellTiter-Glo assay after 72 hours compound treatment. Apoptotic cells were measured by flow cytometry using the Annexin V APC/Sytox Green Kit after 24 hours compound treatment. B, FLT3 protein increases in AMG 925-resistant cells. Left, key FLT3L/FLT3 signaling components were not inhibited by AMG 925. T-FLT3 and P-FLT3 were measured by immunoprecipitation with anti-FLT3 antibody and immunoblotting with anti-FLT3 and anti-P-TYR antibodies. Other proteins were measured by Western blotting directly using their specific antibodies. Right, stabilization of FLT3 proteins in MV4-11^{925R} cells. MV4-11 and MV4-11^{925R} cells were treated with cycloheximide (10 μg/mL) as indicated, and the lysates subjected to immunoprecipitation Western blotting for T-FLT3.

of cycloheximide, no decrease of T-FLT3 in MV4-11^{925R} was observed as in MV4-11, suggesting a stabilization of FLT3 in the MV4-11^{925R} cells.

For comparison with AMG 925, we also isolated sorafenib-resistant MOLM13^{SR} and MV4-11^{SR} clones following a similar protocol. In contrast with MOLM13^{925R} and MV4-11^{925R}, MOLM13^{SR} and MV4-11^{SR} were obtained within 6 weeks and readily adapted to growth in 1.0 μmol/L sorafenib. A secondary FLT3 mutation D835Y was found in all MOLM13^{SR} clones and D835V in all MV4-11^{SR} clones. Both MOLM13^{SR} and MV4-11^{SR} were found to be cross-resistant to AC220 as reported (15), but remained sensitive to AMG 925 (Table 1).

Together, these results indicated that it was harder for the AML cells to develop strong mutational resistance to the FLT3 and CDK4 dual inhibitor AMG 925 than to the FLT3 inhibitor sorafenib.

Isolation of AC220-resistant clones in AML cell lines in the presence or absence of the CDK4 inhibitor PD0332991

To determine whether the inability to isolate strong mutational resistance to AMG 925 was at least partially due to its

CDK4-inhibiting activity, we compared the ability of MV4-11 cells to develop resistance to AC220, a more recently developed FLT3 inhibitor that is also more potent and selective than sorafenib, in the presence or absence of CDK4 inhibitor PD0332991. The concentration of PD0332991 was fixed at 0.001, 0.01, 0.1, and 1 μmol/L whereas AC220 gradually increased from 0.001 to >0.1 μmol/L. Ten independent isolations were performed for each treatment. Within 6 weeks, five clones were obtained from the treatment of AC220 alone, two F691L, one D835V, and two N841K, as identified by sequencing the cDNA of FLT3 kinase domains. For AC220 + PD0332991 treatments, three clones (two D835V and one N841K) were obtained at 0.001 μmol/L PD0332991; one clone (N841K) at 0.01 μmol/L PD0332991; no resistant clones were obtained for 0.1 and 1 μmol/L. The number of resistant clones resulted from different combinations appeared to indicate a dose effect of PD0332991 on the frequency of resistant clones isolated. Proliferation, apoptosis, and PD marker P-STAT5 assays demonstrated a general correlation of these phenotypes characteristic of FLT3 inhibition (Table 1). Despite the small numbers of resistant clones obtained, this result is in support of

Table 1. AMG 925 inhibits growth of AML cells with FLT3 inhibitor-resistant mutations

FLT3 mutations	IC ₅₀ (μmol/L)							
	Cell proliferation inhibition				P-STAT5 inhibition			
	AMG 925	AC220	Sorafenib	PD0332991	AMG 925	AC220	Sorafenib	PD0332991
ITD alone ^a (MOLM13)	0.015 ± 0.002	0.001 ± 0.0003	0.003 ± 0.001	0.089 ± 0.009	0.007 ± 0.001	0.001 ± 0.0003	0.005 ± 0.001	>1
ITD alone ^a (MV4-11)	0.016 ± 0.002	0.001 ± 0.0003	0.004 ± 0.001	0.092 ± 0.008	0.006 ± 0.001	0.001 ± 0.0003	0.004 ± 0.001	>1
ITD/D835Y ^b (MOLM13)	0.028 ± 0.005	0.286 ± 0.011	>1	0.114 ± 0.011	0.011 ± 0.002	0.030 ± 0.003	>1	>1
ITD/D835V ^b (MV4-11)	0.019 ± 0.004	0.258 ± 0.013	>1	0.116 ± 0.011	0.009 ± 0.002	0.028 ± 0.002	0.335 ± 0.012	>1
ITD/D835V ^c (MV4-11)	0.013 ± 0.001	0.128 ± 0.013	0.791 ± 0.036	0.102 ± 0.006	0.013 ± 0.001	0.028 ± 0.007	0.810 ± 0.030	>1
ITD/F691L ^c (MV4-11)	0.073 ± 0.002	0.081 ± 0.055	0.337 ± 0.025	0.112 ± 0.015	0.115 ± 0.005	0.023 ± 0.003	0.334 ± 0.023	>1
ITD/N841K ^c (MV4-11)	0.016 ± 0.002	0.006 ± 0.001	0.125 ± 0.012	0.113 ± 0.002	0.012 ± 0.001	0.010 ± 0.001	0.209 ± 0.005	>1
FLT3 WT (U937)	0.102 ± 0.009	>1	>1	0.102 ± 0.013	>1	>1	>1	>1

NOTE: Names in parentheses indicate AML cell lines hosting the FLT3 mutations. Cell proliferation was determined by CellTiter-Glo assay, P-STAT5 by MSD assay. IC₅₀ values indicated as mean ± SD.

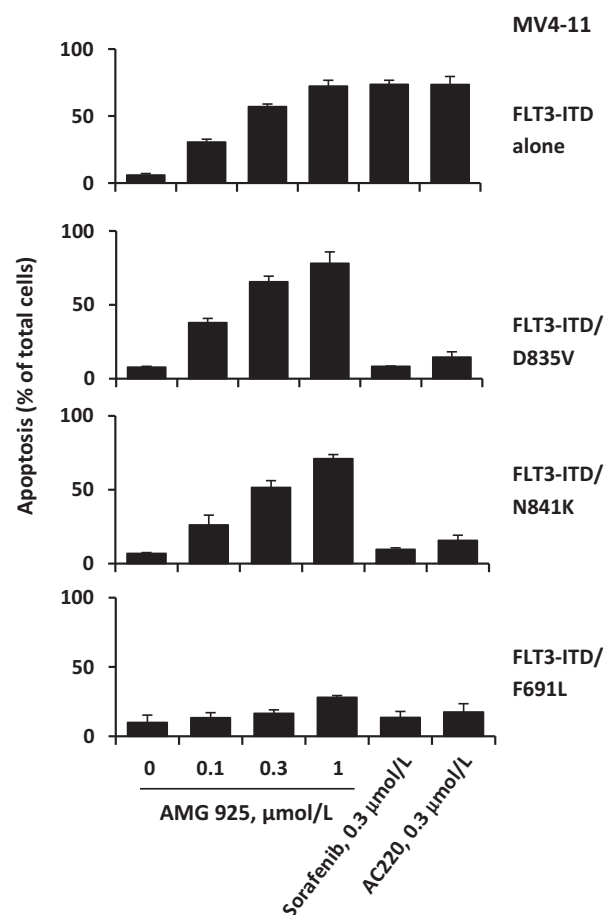
Abbreviation: WT, wild-type.

^aParental cell lines used for isolation of resistant cells.

^bFLT3 mutations isolated by selection with sorafenib.

^cFLT3 mutations isolated by selection with AC220.

our hypothesis that the combination of FLT3 and CDK4 inhibition may make it harder for AML cells to develop resistance compared with inhibition of FLT3 alone.

**Figure 3.**

Induction of apoptosis by AMG 925 in FLT3 inhibitor-resistant AML cells. MV4-11, MV4-11 FLT3-ITD/D835V, MV4-11 FLT3-ITD/N841K, and MV4-11 FLT3-ITD/F691L cells were treated with AMG 925, sorafenib, and AC220 at concentrations as indicated. Cell samples were collected 24 hours after addition of compounds and analyzed for apoptosis by Annexin V/Sytox Green staining and flow cytometry.

All the resistant AML cell clones isolated by AC220 and sorafenib were assayed for cross-resistance by proliferation, P-STAT5, and apoptosis assays, and the results summarized in Table 1 and Fig. 3. AMG 925 potently inhibited D835Y/V, but significantly less so on F691L, consistent with our structural analysis of AMG 925 as a type 1 kinase inhibitor (19).

Profiling of AMG 925 against a panel of FLT3 mutations in Ba/F3 cells

Ba/F3 is a murine lymphoid cell line dependent on IL3 for survival and proliferation. We generated Ba/F3 cell clones stably expressing human FLT3 with ITD mutation in combination with a set of well-known FLT3 mutations (D835Y, F691L, Y842N, and N676K; refs. 15, 34, 35), and tested them for sensitivity to AMG 925 (Table 2). By growth inhibition assay, Ba/F3 clones expressing FLT3-ITD/D835Y, FLT3-ITD/Y842N, FLT3-ITD/N676K, and FLT3-D835Y were similarly sensitive to AMG 925 as the Ba/F3 cells expressing FLT3-ITD alone. All of these mutants were resistant to AC220 and sorafenib to various degrees. The data from Ba/F3 cells expressing FLT3-ITD/D835Y are supportive of our earlier results that AMG 925 inhibited FLT3-ITD/D835Y/V human AML cells that are resistant to sorafenib and AC220 (18). Although Ba/F3 cells expressing FLT3-ITD/F691L showed some resistance to AMG 925, it was worth noting that the resistance to AMG 925 was to a smaller degree compared with that of AC220 and sorafenib. The increase in IC₅₀ values by proliferation assays in the FLT3-ITD/F691L cells compared with the FLT3-ITD cells was 10-, 163-, and 69-fold for AMG 925, AC220, and sorafenib, respectively. The growth inhibition by AMG 925, AC220, and sorafenib correlated well with inhibition of P-STAT5 in the cells, indicating that the growth inhibition by the compounds is largely mediated through FLT3.

Isolation of AMG 925-resistant clones in Ba/F3 cells

For superior clonal growth and tolerance of adverse growth conditions compared with human AML cell lines, the Ba/F3 FLT3-ITD cells described as above were used for isolation of resistance mutations to AMG 925. AC220-resistant FLT3 mutations were also isolated for comparison. Ba/F3 FLT3-ITD cells were treated with mutagen ENU as described previously (36) and plated in 4 × 96-well plates in medium containing AC220 or AMG 925. Selection with 0.05 μmol/L AC220 led to 29 clones (7.6%, Table 3) with various nucleotide changes resulted in amino acid changes at F691, D835, Y842, and N841 in the FLT3 kinase domains. In

Table 2. AMG 925 activity in Ba/F3 cells expressing various FLT3 mutants

FLT3 mutations	IC ₅₀ (μmol/L)					
	Cell proliferation inhibition			P-STAT5 inhibition		
	AMG 925	AC220	Sorafenib	AMG 925	AC220	Sorafenib
Parental Ba/F3 ^a	0.308 ± 0.011	>1	>1	>1	>1	>1
ITD	0.014 ± 0.001	0.001 ± 0.0003	0.006 ± 0.001	0.014 ± 0.001	0.001 ± 0.0003	0.005 ± 0.001
ITD/D835Y	0.011 ± 0.001	0.038 ± 0.003	0.362 ± 0.019	0.011 ± 0.001	0.033 ± 0.002	0.135 ± 0.003
ITD/F691I	0.142 ± 0.011	0.163 ± 0.009	0.416 ± 0.011	0.171 ± 0.009	0.077 ± 0.007	0.195 ± 0.007
ITD/Y842N	0.009 ± 0.001	0.030 ± 0.008	0.341 ± 0.013	0.007 ± 0.001	0.037 ± 0.003	0.132 ± 0.004
ITD/N676K	0.018 ± 0.002	0.028 ± 0.002	0.201 ± 0.013	0.008 ± 0.001	0.030 ± 0.002	0.104 ± 0.009
D835Y	0.014 ± 0.001	0.033 ± 0.003	0.480 ± 0.012	0.022 ± 0.002	0.058 ± 0.002	0.148 ± 0.004

NOTE: Cell proliferation was determined by CellTiter-Glo assay, P-STAT5 by MSD assay. IC₅₀ values indicated as mean ± SD.

^aGrowth of parental Ba/F3 cells is dependent on IL3. Ba/F3 derivatives expressing human FLT3 mutants were made IL3 independent and FLT3 dependent.

contrast, only four AMG 925-resistant clones (1%) were obtained at 0.5 μmol/L AMG 925, all were F691L mutation, two with codon TTT changed to TTA and two to TTG. Selection with higher concentrations of AMG 925 did not lead to resistant clones. Proliferation and P-STAT5 assays confirmed a correlation of resistance with lack of FLT3 inhibition (Supplementary Table S3). This result indicated that under certain conditions, F691 mutations may be isolated by AMG 925, consistent with AMG 925 being a type 1 kinase inhibitor.

Discussion

AMG 925 is an FLT3/CDK4 dual inhibitor recently developed to overcome the resistance issue of selective FLT3 inhibitors by combining FLT3- and CDK4-inhibiting activities in one molecule (18, 19). CDK4 has long been pursued as a genetically validated cancer drug target for a broad spectrum of Rb⁺ cancers, including AML (24, 25, 37). PD0332991 is the first selective CDK4 inhibitor to reach clinical trials (27). A phase II study showed that, in combination with Letrozole (estrogen synthesis inhibitor), PD0332991 prolonged progression-free survival by 18 months in advanced breast cancer patients, providing the first clinical validation of CDK4 as a cancer drug target (27). As a monotherapy, selective CDK4 inhibitors may also encounter resistance issues because CDK4 inhibition is cytostatic and may be relatively easily compensated or bypassed (38, 39). A dual FLT3/CDK4 inhibitor like AMG 925 could have several advantages over FLT3- or CDK4-selective inhibitors alone in treating AML. First, AMG 925 is more efficacious than a CDK4 inhibitor in killing FLT3-ITD AML cells by its FLT3 inhibitory activity. Furthermore, we have confirmed that CDK4 inhibition can enhance the apoptotic effect of an FLT3 inhibitor in FLT3-dependent AML cells with FLT3-ITD. Second, AMG 925 inhibits proliferation of Rb⁺ AML cells with wild-type FLT3. The clinical response in treating AML would be less dependent on ITD allelic burden (10). Third and more importantly, by targeting two kinases whose functions are not fully overlapping but both crucial for AML cell growth, AMG 925 may reduce the chances of FLT3 resistance mutations and lead to more durable clinical responses than FLT3 inhibitors alone. It is tempting to draw an analogy here between FLT3-dependent AML

and estrogen-dependent breast cancer. The growth factor dependence in both cases could reflect the relative integrity of growth regulation pathways in the cancer cells (e.g., relatively low apoptotic potential and high dependence on CDK4). This is thought to be important for the maximal antiproliferation efficacy of a CDK4 inhibitor, and preventing the development of resistance to inhibitors of growth factor signaling. In clinical studies, CDK4 inhibitor PD0332991 appeared better tolerated than standard care of chemotherapy. Therefore, compared with the combination of FLT3 inhibitors with chemotherapies (40, 41), AMG 925, which combines FLT3 with CDK4-inhibiting activities, may show a better clinical toxicity profile and would be a preferable treatment for older patients with AML (27).

To demonstrate that AMG 925 may prevent FLT3 inhibitor resistance mutations, we attempted to isolate AMG 925-resistant clones in the AML cell lines MOLM13 and MV4-11. We found that the development of resistance to AMG 925 was rather different from that of the type 2 FLT3 inhibitors sorafenib and AC220. The FLT3 inhibitor-resistant clones could be fairly readily isolated within 6 weeks. The resistance was strong with IC₅₀ values increased over 100- to 200-fold in cell proliferation assays compared with those in the parental cells. In contrast, it took 8 months of selection for establishment of clones that were only partially resistant to AMG 925, with IC₅₀ values increased by only 3- to 5-fold compared with those of the parental cells. The AMG 925-resistant clones grew slower with a doubling time of 72 versus 48 hours for the parental cells. The resistance to AMG 925 was reversible and no secondary resistance mutations were identified in FLT3, indicating an epigenetic responses to the drug treatment. An increase in total FLT3 was found in the AMG 925-resistant AML cells and believed to be the major mechanism of the resistance. The similar characteristics of all of the AMG 925-resistant clones from two AML cell lines may indicate that this may represent the primary resistance mechanism to the treatment conditions tested. We also tried several rounds of selection for AMG 925-resistant clones, starting with concentrations of AMG 925 higher than 0.001 μmol/L. No resistant clones were able to grow out. Together, these results are supportive of the hypothesis that simultaneously inhibiting FLT3 and CDK4 in AML cells may inhibit development of drug resistance as occurs with FLT3 inhibitors alone.

Table 3. Resistance mutations in FLT3 acquired through growing the Ba/F3 FLT3-ITD cells in the presence of AC220 or AMG 925

Compound	Number of secondary resistance mutations in FLT3-ITD								Isolation frequency
	F691L	Y842N	Y842H	Y842C	Y842D	N841K	D835V	D835Y	
AC220	14	4	2	3	2	2	1	1	7.6%
AMG 925	4	0	0	0	0	0	0	0	1.0%

NOTE: Mutagenized Ba/F3 FLT3-ITD cells were seeded into 4 × 96-well plates with growth medium containing AC220 or AMG 925. Resistant cell clones were isolated and the genomic DNA was sequenced for secondary mutations in FLT3.

To support the hypothesis that the CDK4-inhibiting activity of AMG 925 accounted for our inability to isolate mutations resistant to the compound, we tried to isolate resistant mutations to the FLT3 inhibitor AC220 in the presence or absence of the CDK4 inhibitor PD0332991. With AC220 alone, we were able to isolate five AC220-resistant clones with one having mutation D835V, two F691L, and two N841K. With AC220 combined with PD0332991, four mutations were obtained, three (two D835V and one N841K) with 0.001 $\mu\text{mol/L}$ PD0332991, and one (N841K) with 0.01 $\mu\text{mol/L}$ PD0332991. No resistant clones grew out when PD0332991 was increased to 0.1 $\mu\text{mol/L}$ or higher. These results indicated that sufficiently high concentrations of PD0332991 will prevent FLT3 inhibitor-resistant mutations from occurring in a dose-dependent manner. The cell growth inhibition IC_{50} value of PD0332991 in MV4-11 cells was 0.092 $\mu\text{mol/L}$. Therefore, it appeared that a concentration of PD0332991 around its IC_{50} value would be needed to significantly reduce resistant mutations. Only D835 mutations were also isolated with sorafenib in our earlier attempt. F691L and N841K were additional ones obtained with AC220. The reason for the difference could be due to a difference in potency and selectivity of the compounds, but could also be due to the difficulty in achieving closely similar conditions for isolation process with AML cells.

Another feature of AMG 925 that might also contribute to the inability to isolate resistant mutations in FLT3 is that the compound inhibits FLT3-ITD/D835 mutants. D835 represents one of the two hot spot mutations in FLT3 that confer resistance to AC220 and sorafenib (15, 42). Structural analyses of the binding of AMG 925, sorafenib, and AC220 to FLT3 suggest that AMG 925 is a type 1 kinase inhibitor whereas sorafenib and AC220 are type 2 inhibitors (15, 19). D835 was shown to be critical in interaction with sorafenib and AC200 but not for AMG 925. These analyses explain and validate our observations of the cellular activity differences in inhibiting various FLT3 mutants by these compounds.

FLT3 mutants with amino acid changes at the F691 gatekeeper residue were expected to affect binding to both type 1 and 2 FLT3 inhibitors. We were able to isolate F691 mutation (F691L) with AC220 but not with AMG 925 in AML cell lines. As shown in Table 1, the AC220-resistant MV4-11-bearing FLT3-ITD/F691L was indeed about 5- and >10-fold more resistant to AMG 925 than the parental MV4-11 by proliferation assays and P-STAT5 assays, respectively. Our inability to isolate F691 mutations in MV4-11 with AMG 925 was thought primarily due to the CDK4-inhibiting activity of the compound. Another possibility could be that the AML cell lines adapted to growth at lower concentrations of AMG 925 relatively easily through increasing expression of the FLT3 protein. It is conceivable that more variations in isolation protocols might have resulted in the isolation of F691 mutations with AMG 925 in AML cell lines.

The Ba/F3 cell line is known to be easier to work with for the isolation of mutations resistant to kinase inhibitors (43). Therefore, we also tried to isolate AMG 925-resistant clones using this cell line with AC220 as a reference. It is worth noting that these mouse cells are generally 5- to 10-fold less sensitive to CDK4 inhibitors compared with human cells (our unpublished data; ref. 44). For this reason, it was thought that it would be easier to isolate mutations at gate keeper residue F691 with AMG 925 in this mouse system. We obtained numerous resistant mutations to AC220, including the two hot spot mutations at D835 and F691

(Table 3), which demonstrates that the isolation protocol worked well. As expected, we were able to isolate 4 AMG 925-resistant clones, all having mutation F691L. Although the number of AMG 925-resistant mutations is small for a firm conclusion, this result is consistent with AMG 925 being a type 1 kinase inhibitor. The clinical implication would be that mutations at F691 might arise during the treatment of AMG 925 when CDK4 target coverage would be significantly compromised for various reasons (45). It is worth mentioning that the resistance to AMG 925 conferred by F691L was relatively weak compared with resistance to AC220 and sorafenib, implying that such mutations might not as easily occur in AMG 925 treatment.

Although we believe that the CDK4 activity of AMG 925 and its type 1 kinase inhibitor characteristics are primarily responsible for the lack of isolation of FLT3 mutations in AML cells, we could not rule out the possibility of some role by other activities associated with AMG 925. However, the proliferation IC_{50} value of AMG 925 in Rb^- cells, including MDA-MB-436, is in the $\mu\text{mol/L}$ range. Rb^- solid tumor cells are independent of the FLT3 and CDK4 pathways. The high IC_{50} value in these cells indicates a fairly low nonspecific cellular activity of AMG 925. In addition, the successful isolation of F691L mutations in the Ba/F3 system also indicated that it was unlikely that nonspecific activities of AMG 925 were playing a major role in the inability to isolate AMG 925-resistant cells in AML cells. It is possible that the inability of AMG 925-resistant cells to grow at higher concentrations of AMG 925 may be partially due to the nonspecific activities of the compound.

In summary, mutational resistance has been a serious clinical issue for FLT3 inhibitors. Resistance to AMG 925, a dual FLT3/CDK4 inhibitor, appears harder to develop in AML cell lines than that to the FLT3 inhibitors. The results of the treatment of the AML cell line MV4-11 with a combination of AC220 and PD0332991 support the hypothesis that the CDK4-inhibiting activity of AMG 925 played a significant role in reducing the appearance of FLT3 resistance mutations. In addition, AMG 925 as a type 1 kinase inhibitor, potently inhibited FLT3-ITD/D835, one of the major resistance mutations acquired during AC220 treatment. We believe that AMG 925 has the potential to overcome drug resistance associated with FLT3 inhibitors and could lead to more durable clinical responses in patients with AML. Clinical trials in patients with AML are currently being planned.

Disclosure of Potential Conflicts of Interest

L.R. McGee, K. Newhall, A. Kamb, and D. Wickramasinghe have ownership interest (including patents) in Amgen, Inc. A. Sinclair reports receiving a commercial research grant from and has ownership interest (including patents) in Amgen, Inc. No potential conflicts of interest were disclosed by the other authors.

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AMG 925 Is a Dual FLT3/CDK4 Inhibitor with the Potential to Overcome FLT3 Inhibitor Resistance in Acute Myeloid Leukemia

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