



Published in final edited form as:

Leukemia. 2016 June ; 30(6): 1422–1425. doi:10.1038/leu.2015.320.

Quizartinib elicits differential responses that correlate with karyotype and genotype of the leukemic clone

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Fms-like tyrosine kinase 3 (*FLT3*) is one of the most commonly mutated genes in acute myeloid leukemia (AML) and as such has been a highly studied therapeutic target in the field. Indeed, highly potent *FLT3* inhibitors such as quizartinib (AC220) have generated substantial antileukemic clinical effects among relapsed/refractory AML patients with *FLT3*-internal tandem duplication (ITD) mutations, as well as a smaller number of patients without documented *FLT3* mutation. Importantly, nearly 70% of quizartinib-treated *FLT3*-ITD+ patients show objective antileukemic responses per the modified International Working Group response criteria, many of which allowed subsequent allogeneic hematopoietic cell transplantation and prolonged survival in some.

Although initially *FLT3* inhibition was believed to act on myeloblasts primarily via direct cytotoxicity, recent data suggest that in some AML patients *FLT3* inhibition causes terminal differentiation of *FLT3*-ITD+ blasts. As *FLT3* inhibition is a major area of ongoing preclinical and clinical investigation in the field of AML, we sought to define predictors of

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Results were presented in part at the 54th annual meeting of the American Society of Hematology meeting.

CONFLICT OF INTEREST

AEP has served as a consultant to Ambit Biosciences, Astellas Pharmaceuticals and Arog Pharmaceuticals. CCS received clinical trial grant funding from Astellas. The remaining authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GEN designed research, collected data, performed research, performed histopathologic analysis of clinical samples and wrote the paper; JC analyzed data and wrote the paper; DR collected data; JDM and CDW performed research NPS and CCS performed research; AB performed histopathologic analysis of clinical samples; MC and AEP designed research, analyzed data and wrote the paper. All authors provided critical review of the paper and approved the submission.

Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)

differentiation and cytotoxic effects of quizartinib therapy at a molecular and genetic level. We retrospectively performed an independent analysis of all marrow studies from the 21 patients treated with quizartinib at our center on a recent phase 2 clinical trial. Here we confirm that *FLT3* inhibition by quizartinib generates potent antileukemic effects manifesting as two major patterns of drug response, namely differentiation and cytotoxic responses. We also provide evidence that cooperating mutations modify clinical response to *FLT3* kinase inhibition.

Patients with relapsed/refractory AML after one to two prior lines of chemotherapy were enrolled in a phase 2 study of quizartinib monotherapy at the Hospital of the University of Pennsylvania (NCT00989261). We report here a correlative analysis of the entire institutional cohort that enrolled to the larger multicenter NCT00989261 phase 2 trial, internally termed the AC220-002, or 'ACE' trial. All patients provided written informed consent according to the Declaration of Helsinki both for trial participation as well as for institutional tissue banking. Bone marrow aspirates were collected at baseline and on treatment days 15, 29 and 56, unless a protocol-defined complete remission CRp, or CRi occurred. Marrow samples were additionally collected at study withdrawal or disease progression. All available histomorphologic, cytogenetic and molecular genetic materials were independently reviewed for publication. Comparison between patients was done at the best response time points defined by the lowest percent blasts in the bone marrow assessment. Bone marrow biopsies and aspirates were analyzed by light microscopy using a Leica DM 2500 clinical microscope (Buffalo Grove, IL, USA). Photographs were taken using an attached Leica DFC420 digital camera (Buffalo Grove, IL, USA). Fisher's exact test was used in the analysis of contingency tables.

Twenty-one patient samples were analyzed, of which 19 were *FLT3*-ITD+ at initial diagnosis (the trial eventually expanded to enroll patients lacking *FLT3*-ITD). Among four patients with only wild-type (WT) *FLT3* detected at study entry, two were previously *FLT3*-ITD+ but had low blast percentages at screening (<20% but >5% blasts consistent with early relapse). Therefore, the lack of *FLT3*-ITD detection in these cases may reflect sensitivity limitations of assays or loss of *FLT3*-ITD mutation at relapse.

Objective antileukemic treatment effects were seen in 20/21 patients, evident by rapid clearance of peripheral blood blasts and extramedullary leukemia (for example, leukemia cutis), typically within 1 week, consistent with previously published trial data (data not shown). Primary resistance to quizartinib was only observed in one patient who in addition to a low-abundance *FLT3*-ITD (ratio of *FLT3*-ITD:WT of 1:14) had *NRAS*G13D, which has been shown to confer in vitro resistance to *FLT3* inhibitors. On serial marrow testing, reductions in marrow blasts to <50% of baseline blast percentage were noted in 18/19 patients (one patient was not evaluable owing to <10% marrow blasts at baseline marrow; Figure 1a).

Reviewing the patients' marrows, we noted that responses to quizartinib separated into two different groups. In what we term a cytotoxic response, quizartinib rapidly reduced marrow cellularity (to <40%) and peripheral counts showed limited, if any neutrophil recovery after blast clearance. In the contrasting response, which we term a differentiation response,

patients retained or developed increased marrow cellularity and blast clearance from the blood was followed by robust neutrophil recovery, generally on the second month of therapy (Figure 1b). Representative photomicrographs of both responses in the bone marrow are shown in Figures 1c and d. Serial marrow sampling of patients with differentiation response generally showed hypercellularity that was preserved across 2–3 months of study therapy (Supplementary Table 1). Progression of leukemia or withdrawal from study therapy for allogeneic transplant limited longer follow-up of these marrow responses.

Importantly, in patients with the differentiation response, despite a marked reduction in marrow blast percentages, the allelic ratio of *FLT3*-ITD:WT remained relatively constant in many patients (Figure 1e), suggesting that regenerating hematopoiesis in these cases reflected differentiation of the leukemic clone. Indeed, before quizartinib, *FLT3*-ITD could only be demonstrated in the peripheral blood in the presence of circulating blasts. However, following clearance of circulating blasts in response to quizartinib, *FLT3*-ITD was readily detected in purified circulating neutrophils from patients with differentiation response (Supplementary Table 2). Allelic ratios of *FLT3*-ITD:WT from marrow obtained during differentiation response also generally showed preservation of *FLT3*-ITD allelic burden comparable to pretreatment levels, despite reduction in marrow blasts to <5%.

Next, we observed that nearly all 9 patients with the differentiation response pattern (8/9 *FLT3*-ITD+) had a normal karyotype, whereas conversely all 10 of the patients with a cytotoxic response (7/10 *FLT3*-ITD+ at study enrollment) had a baseline abnormal karyotype upon study enrollment (Table 1). We note that karyotypes listed reflect those measured at study entry and abnormal karyotypes seen then were in some cases normal at original AML diagnosis. We next tested whether the differentiation and cytotoxic responses differed with regard to particular cooperating mutations. As shown in Table 1, targeted next-generation sequencing of 33 hematologic malignancy-associated genes revealed that 8/9 patients with differentiation response to quizartinib had mutated *DNMT3A* (5 R882, 3 pathogenic non-R882 substitutions and 1 variant of unclear significance), and 7/9 additionally had an *NPM1* mutation. By contrast, a diverse number of mutations were seen among the 8 patients with cytotoxic response who had samples available for mutational testing, including 2 with *DNMT3A* mutations, 1 of which also contained an *NPM1* mutation. The latter patient had abnormal cytogenetics as shown. Noteworthy, at progression, 5/8 patients demonstrated new *FLT3*-TKD (D835) mutations by PCR and/or direct sequencing, 3 after cytotoxic response and 2 after differentiation response. No difference in time to progression was noted in the two groups of patients.

To summarize, AC220 induces two different patterns of clinical response to therapy. Patients demonstrating a differentiation response are frequently characterized by normal cytogenetics and the presence of *FLT3*-ITD, *DNMT3A*, and *NPM1* mutations. Overall, our data suggest that *FLT3*-ITD potentially serves different functions in leukemogenesis based on the context of various cooperating mutations. Mutational analysis by the Cancer Genome Atlas revealed a subgroup of AML that contain mutations in *FLT3*, *DNMT3A* and *NPM1*. The significance of this grouping of mutations remains unexplained. *DNMT3A* catalyzes the methylation of cytosine residues at 5-carbon sites, thereby epigenetically altering gene expression. Recurrent mutations in *DNMT3A* disrupt its methyltransferase activity, occur early in

leukemogenesis, and expand the hematopoietic stem cell pool; however, functional effects upon differentiation are controversial. *NPM1* contributes to ribosome biogenesis, cell division and DNA damage response/repair; however, its precise function in normal hematopoiesis or leukemogenesis remains incompletely understood. Our data provide the first demonstration that this group of patients have a unique differentiation response to AC220, which may suggest the underlying biology of this triple-mutant leukemia affecting myeloid differentiation. Our study sample was highly enriched for the mutation composite of *FLT3*, *DNMT3A* and *NPM1*. Of note, patients with this triple mutational profile appear to have an increased likelihood of relapse, which may explain their frequency in our cohort of relapsed/refractory patients (Papaemmanuil E, et al. EHA abstracts 2015, and personal communication). Because of this enrichment, effects generated by *NPM1* or *DNMT3A* alone could be missed, as is the potential to detect other genotypes associated with differentiation response. This may have the potential to bias our results, and further study is needed to validate our observations.

Taken together, data from our cohort provide additional mechanistic insight into the antileukemic effects of potent *FLT3* inhibition in AML patients by providing a cytogenetic and molecular correlation between the differential cytotoxic and differentiation effects experienced by patients on quizartinib. In addition, the data may generate discussion regarding possibly redefining treatment responses to *FLT3* and other molecularly targeted inhibitors in AML. There is precedent for this in other tumor types where clinical benefit to molecularly targeted agents has been noted in the absence of traditional metrics. This is exemplified by ibrutinib therapy of chronic lymphocytic leukemia, where therapy-induced lymphocytosis is characteristic of response rather than resistance. Although determining clinical benefit to *FLT3* inhibitors is beyond the scope of this report, our data nonetheless provide a framework for predicting and understanding patient responses to *FLT3* inhibition both as monotherapy and future combination trials.

Acknowledgments

This study was supported by the National Institutes of Health, the National Cancer Institute (NCI; grant 1K23CA141054 to AEP) and the Leukemia and Lymphoma Society (NPS, CCS and AEP). NPS was supported by a grant from the National Cancer Institute (1R01 CA166616-01) and MC is supported by R21CA198621 also from the NCI. We thank Cezary Swider, PhD for technical expertise and assistance with cell processing.

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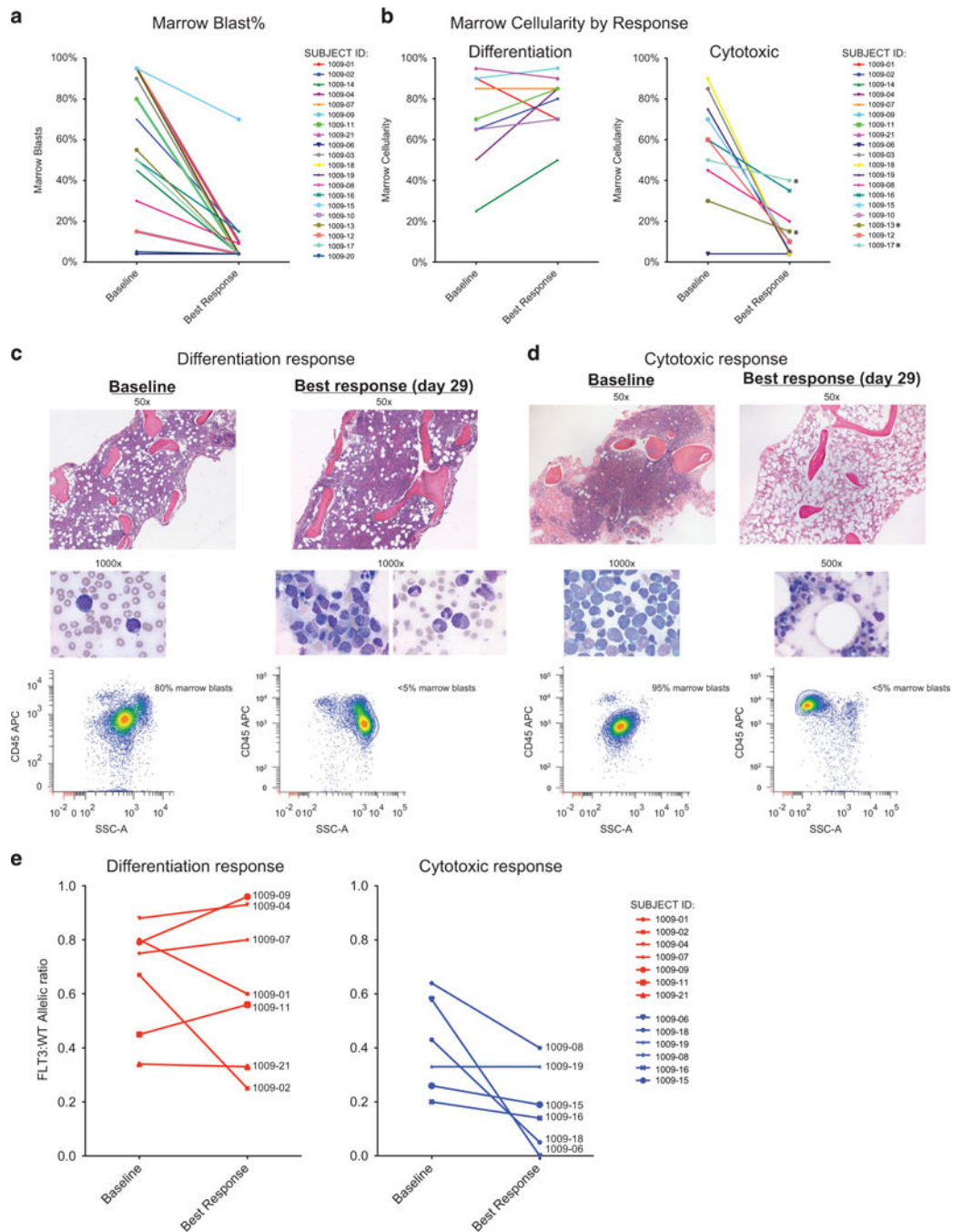


Figure 1. Quizartinib therapy reduces marrow blast burden by the induction of either differentiation or cytotoxicity responses. **(a, b)** Comparison of blast burden and marrow cellularity at baseline and at best response time points. **(c, d)** Representative photomicrographs of two patients with differentiation and cytotoxic responses. **(e)** Graph of *FLT3*-ITD:WT allelic ratio, organized by color according to response. The ratio has been converted to ITD frequency (ITD/ITD+WT) for ease of graphical display.

Table 1
DNMT3A mutation status segregates patients into differentiation and cytotoxic responses

Subjec	<i>FLT3</i> Genotype	Best marrow blast (%)	Baseline karyotype response	Karyotype at response	Cooperating mutations
<i>Differentiation response</i>					
1009-01	<i>FLT3</i> -ITD	10	46,XY	46,XY	<i>DNMT3A</i> *, <i>NPM1</i> , <i>ASXL1</i> , <i>IDH1</i>
1009-02	<i>FLT3</i> -ITD	15	46,XX	46,XX	<i>DNMT3A</i> , <i>NPM1</i> , <i>TET2</i>
1009-14	<i>FLT3</i> -ITD	<5	46,XY	46,XY	<i>DNMT3A</i> , <i>NPM1</i>
1009-04	<i>FLT3</i> -ITD	<5	46,XX	46,XX	<i>DNMT3A</i> , <i>NPM1</i> , <i>WT1</i> , <i>ATM</i> *
1009-07	<i>FLT3</i> -ITD	<5	46,XX	46,XX	<i>DNMT3A</i> , <i>NPM1</i>
1009-09	<i>FLT3</i> -ITD	<5	46,XY	46,XY	<i>DNMT3A</i> , <i>NPM1</i> , <i>TET2</i>
1009-11	<i>FLT3</i> -ITD	<5	46,XX	46,XX	<i>DNMT3A</i> , <i>NPM1</i> , <i>TET2</i>
1009-10	<i>FLT3</i> -WT	<5	46,XY	46,XY	<i>TET2</i>
1009-21	<i>FLT3</i> -ITD	10	46,XY,+11	46,XY,+11	<i>DNMT3A</i> , <i>ASXL1</i>
<i>Cytotoxic response</i>					
1009-06	<i>FLT3</i> -ITD	<5	Complex	46,XY	Not evaluable
1009-03	<i>FLT3</i> -ITD	<5	Hyperdiploid/complex	46,XX	<i>DNMT3A</i> , <i>RUNX1</i>
1009-13	<i>FLT3</i> -WT	<5	Complex	46,XY	<i>TP53</i> , <i>NOTCH1</i>
1009-12	<i>FLT3</i> -WT	<5	Complex	46,XY	Not evaluable
1009-17	<i>FLT3</i> -WT	<5	Complex	Complex	<i>TP53</i> , <i>JAK2</i>
1009-19	<i>FLT3</i> -ITD	<5	Complex	No growth	No mutations
1009-08	<i>FLT3</i> -ITD	<10	Complex	Complex	<i>RUNX1</i>
1009-18	<i>FLT3</i> -ITD	<5	46,XY,t(8;21)(q22;q22) with multiple abnormalities	46,XY,t(8;21)(q22;q22) with multiple additional abnormalities	<i>TET2</i> *
1009-16	<i>FLT3</i> -ITD	15	46,XY,del(5)(q23q33)	46,XY,del(5)(q23q33)	<i>ATM</i> *
1009-15	<i>FLT3</i> -ITD	70	47,XX,+8,del(16)(q13)	47,XX,+8,del(16)(q13)	<i>DNMT3A</i> , <i>NPM1</i>

Abbreviations: *FLT3*, Fms-like tyrosine kinase 3; ITD, internal tandem duplication. Genetic and cytogenetic studies at baseline and best response are shown. Responding patients with normal karyotype uniformly showed differentiation response. Patients with differentiation response had a statistically significant increase in the incidence of *NPM1* mutations (odds ratio (OR) = 24.5, *P*<0.01) and/or *DNMT3A* (OR = 10.5, *P*<0.05, Fisher's exact test). One subject with rapidly progressive leukemia and one with unclassifiable response due to low baseline blast content are not shown. *FLT3*-ITD and D835 mutations were detected using fluorescent primers and multiplexed PCR amplification followed by capillary electrophoresis and/or EcoRV digestion resistance assay by published methods. Allelic ratios of *FLT3*-ITD to the WT sequence were calculated using peak area (GeneMapper Software, Applied Biosystems, Grand Island, NY, USA). DNA was additionally extracted from cryopreserved blast samples and analyzed by next-generation sequencing for 23 genes commonly mutated in AML. Briefly, sequencing was done using an amplicon-based capture protocol (Luminex, Grand Island, NY, USA) with sequencing using MiSeq. Average depth of coverage was over 2000X and minimum depth was 250X. Sensitivity for mutations was 4% for all mutations other than *FLT3*-ITD, which was sensitive to a variant allele frequency of 1%. With the exception of variants of unclear significance (VOUS)—as indicated by asterisk—all gene mutations listed are recurrent pathogenic sequence alterations as

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referenced by public databases, including dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), the Catalog of Somatic Mutations in Cancer (<http://cancer.sanger.ac.uk/cosmic/>) and the 1000 Genomes Project (<http://www.1000genomes.org/>). Statistical analyses did not include YOUS.