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1 # *A noncanonical FLT3 gatekeeper mutation disrupts gilteritinib binding and*
2 # *confers resistance*

3 Sunil K. Joshi[1,2,3], Setareh Sharzehi[1,3,4], Janét Pittsenbarger[1,3], Daniel Bottomly[1,5], Cristina E.
4 Tognon[1,3], Shannon K. McWeeney[1,5], Brian J. Druker[1,3,4], Elie Traer[1,3,4]
5
6 [1]Knight Cancer Institute, Oregon Health & Science University, Portland, OR
7 [2]Department of Physiology & Pharmacology, School of Medicine, Oregon Health & Science
8 University, Portland, OR
9 [3]Division of Hematology & Medical Oncology, Department of Medicine, Oregon Health & Science
10 University, Portland, OR
11 [4]Department of Cell, Development, & Cancer Biology, Oregon Health & Science University,
12 Portland, OR
13 [5]Division of Bioinformatics and Computational Biology, Department of Medical Informatics and
14 Clinical Epidemiology, Oregon Health & Science University, Portland, OR
15
16 [§]**CORRESPONDENCE:** Elie Traer, M.D., Ph.D. (traere@ohsu.edu); Oregon Health & Science
17 University, 3181 SW Sam Jackson Park Road, Mail Code: KR-HEM, Portland, OR 97239; Phone:
18 503.494.3553; Fax: 503.494.3465
19
20 **RUNNING TITLE:** *FLT3^{N701K} confers gilteritinib resistance*
21
22 **KEYWORDS:** AML, FLT3, Gilteritinib, Drug Resistance, Resistance point mutation, Tyrosine
23 Kinase Inhibitor
24
25

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26 **ABSTRACT:**

27 The recent FDA approval of the FLT3 inhibitor, gilteritinib, for AML represents a major
28 breakthrough for treatment of FLT3 mutated AML. However, patients only respond to gilteritinib
29 for 6-7 months due to the emergence of drug resistance. Clinical resistance to gilteritinib is often
30 associated with expansion of NRAS mutations, and less commonly via gatekeeper mutations in
31 FLT3, with F691L being the most common. We developed an *in vitro* model that charts the
32 temporal evolution of resistance to gilteritinib from early microenvironmental-mediated resistance
33 to late intrinsic resistance mutations. Our model system accurately recapitulates the expansion of
34 NRAS mutations and the F691L gatekeeper mutations found in AML patients. As part of this
35 study, we also identified a novel FLT3^{N701K} mutation that also appeared to promote resistance to
36 gilteritinib. Using the Ba/F3 system, we demonstrate that N701K mutations effectively act like a
37 gatekeeper mutation and block gilteritinib from binding to FLT3, thereby promoting resistance.
38 Structural modeling of FLT3 reveals how N701K, and other reported gilteritinib resistance
39 mutations, obstruct the gilteritinib binding pocket on FLT3. Interestingly, FLT3^{N701K} does not block
40 quizartinib binding, suggesting that FLT3^{N701K} mutations are more specific for type 1 FLT3
41 inhibitors (gilteritinib, midostaurin, and crenolanib). Thus, our data suggests that for the FLT3^{N701K}
42 mutation, switching classes of FLT3 inhibitors may restore clinical response. As the use of
43 gilteritinib expands in the clinic, this information will become critical to define clinical strategies to
44 manage gilteritinib resistance.

45

46 **INTRODUCTION:**

47 Acute myeloid leukemia (AML) is a genetically heterogenous disease with approximately
48 20,000 new cases per year in the United States[1, 2]. Patients with AML have a 5-year survival of
49 <25%, and intense efforts are underway to develop new treatments to improve survival[1].
50 Mutations in the FMS-like tyrosine kinase-3 (FLT3) gene are among the most common genomic
51 aberrations in AML. Internal tandem duplication (ITD) in the juxtamembrane domain of FLT3 are

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52 present in approximately 20% of patients with AML. These mutations cause constitutive kinase
53 activity, and lead to an increased risk of relapse and reduced survival. Another set of mutations
54 in the tyrosine kinase domain (TKD) of FLT3 occur in 5-10% of AML patients. In contrast to FLT3-
55 ITD, FLT3 TKD mutations result in less activation of FLT3 and do not increase the risk of relapse[3].
56 Multiple FLT3 tyrosine kinase inhibitors have been developed and can be separated into two
57 classes. Type I inhibitors are canonical ATP competitors that bind the ATP binding site of FLT3
58 in the active conformation and are effective against both ITD and TKD mutations. By contrast,
59 type II inhibitors bind the hydrophobic region adjacent to the ATP binding domain in the inactive
60 conformation. Type II inhibitors are effective against FLT3-ITD, but do not inhibit FLT3 TKD
61 mutations. Quizartinib, a type II inhibitor, has potent activity against FLT3, KIT, and RET. Despite
62 high response rates as a monotherapy in patients with relapsed/refractory AML, the duration of
63 response to quizartinib is approximately 4 months, and resistance via FLT3 TKD mutations is
64 common[4-6]. These mutations occur frequently at the activation loop residue D835 and less
65 commonly at F691 which represents the "gatekeeper" position in FLT3[4].
66 Gilteritinib is second-generation inhibitor that targets FLT3 and AXL[7]. As a type I inhibitor, it
67 is active against TKD mutations that impart quizartinib resistance. It was approved as
68 monotherapy in relapsed/refractory patients with AML based upon the randomized phase 3
69 clinical study (ADMIRAL) which compared gilteritinib with chemotherapy[7]. Despite the significant
70 survival benefit in the gilteritinib arm, monotherapy is limited by the development of resistance,
71 which typically occurs after 6-7 months. Resistance to gilteritinib most commonly occurs through
72 acquisition/expansion of NRAS mutations, however a minority of patients with F691L gatekeeper
73 mutations were also identified[8]. To search for additional resistance mutations to gilteritinib, Tarver
74 *et al.* used a well-established ENU mutagenesis assay and identified Y693C/N and G697S as
75 mutations that confer resistance *in vitro*[6]. These mutations appear to function similar to the
76 gatekeeper mutation by blocking gilteritinib binding to FLT3, but have not been reported in
77 patients.

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78 **RESULTS & DISCUSSION:**

79 To more broadly investigate mechanisms of resistance to gilteritinib, we developed a two-step
80 model of resistance that recapitulates the role of the marrow microenvironment (**Figure 1A**). In
81 the first stage of resistance, or early resistance, the FLT3-mutated AML cell lines MOLM14 and
82 MV4;11 are cultured with exogenous ligands, fibroblast growth factor 2 (FGF2) and FLT3 ligand
83 (FL), that are normally supplied by marrow stromal cells. These culture conditions allow the cells
84 to become resistant to gilteritinib without the need for resistance mutations[9]. When ligands are
85 removed, the cells regain sensitivity to gilteritinib, but ultimately become resistant, which we term
86 late resistance. At this point, intrinsic resistance mutations were identified in all of the cultures via
87 whole exome sequencing. Similar to clinical data[7], we found that the most common mutations are
88 activating mutations in NRAS[10]. One late resistant culture had an $FLT3^{F691L}$ gatekeeper mutation,
89 and 3 cultures had an $FLT3^{N701K}$ mutation, which has not previously been reported (**Figure 1B**).
90 Given its proximity to F691L (**Figure 1C-D**), we hypothesized that this mutation might also disrupt
91 gilteritinib binding to FLT3.

92 To determine whether the $FLT3^{N701K}$ mutation has oncogenic capacity, we evaluated this
93 mutation in the Ba/F3 transformation assay. Ba/F3 cells are normally IL-3 dependent but the
94 presence of certain oncogenes transforms them to grow indefinitely in the absence of IL-3[11]. The
95 $FLT3^{N701K}$ mutation, similar to $FLT3^{ITD}$ and $FLT3^{D835Y}$, is an activating mutation and promoted
96 growth of Ba/F3 cells in the absence of IL-3, whereas the parental, empty vector, FLT3 wild type
97 ($FLT3^{WT}$), or $FLT3^{F691L}$ did not confer IL-3-independent growth (**Figure 1E**).

98 In contrast to Ba/F3 cells expressing $FLT3^{D835Y}$, Ba/F3 cells with $FLT3^{N701K}$ were much less
99 sensitive to gilteritinib with an approximate 8.5-fold increase in IC_{50} (**Figure 2A**). To test whether
100 $FLT3^{N701K}$ also promoted resistance to gilteritinib in the presence of $FLT3^{ITD}$ mutations (**Figure**
101 **1B**), we generated $FLT3^{ITD + N701K}$ and $FLT3^{ITD + F691L}$ double mutants and expressed them in Ba/F3
102 cells. Concordant with previous studies[4], the $FLT3^{ITD + F691L}$ mutant demonstrated an approximate
103 11-fold increase in IC_{50} to gilteritinib compared to FLT3-ITD alone. $FLT3^{ITD+N701K}$ Ba/F3 cells were

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nearly identical to FLT3$^{ITD + F691L}$ cells in their resistance to gilteritinib (**Figure 2B**). As a control, FLT3^{WT} Ba/F3 cells grown with IL-3 were insensitive to gilteritinib at comparable doses.

Next, we assessed the impact of FLT3^{N701K} mutations on downstream FLT3 signaling pathways. Ba/F3 cells transformed with FLT3^{N701K}, FLT3^{ITD}, FLT3$^{ITD + F691L}$, and FLT3$^{ITD + N701K}$ all resulted in phosphorylation of FLT3 (Y589/591) and STAT5 (Y694), AKT (S473), and ERK (T202/Y204) (**Figure 2C**). However, only FLT3$^{ITD + N701K}$ or FLT3$^{ITD + F691L}$ showed sustained phospho-FLT3 with increasing concentrations of gilteritinib (**Figure 2D**), indicating that both of these mutations prevent gilteritinib inhibition of FLT3, particularly at lower doses. The FLT3 kinase activity as reflected by FLT3 phosphorylation mirrored the viability assays in **Figure 2B**.

Since F691L gatekeeper mutations are known to drive resistance to multiple FLT3 inhibitors[4, 8, 12, 13], we treated FLT3^{ITD}, FLT3$^{ITD + N701K}$ and FLT3$^{ITD + F691L}$ Ba/F3 cells with midostaurin, crenolanib, and quizartinib. Although FLT3$^{ITD + F691L}$ and FLT3$^{ITD + N701K}$ were largely insensitive to type I inhibitors midostaurin and crenolanib, cells with FLT3$^{ITD + N701K}$ were notably more sensitive to the type II inhibitor quizartinib (**Supplemental Figure 1**), suggesting that N701K blocks gilteritinib binding of type I inhibitors more effectively than type II. This was further apparent from our modeling of the FLT3^{N701K} mutation. While the FLT3^{N701K} mutation may sterically interfere with the binding of gilteritinib, quizartinib binding does not appear to be affected (**Supplemental Figure 2**).

Through our studies, we identified the novel FLT3^{N701K} mutation in addition to the FLT3^{F691L} gatekeeper mutation. We used the Ba/F3 system to demonstrate that N701K blocks gilteritinib binding to FLT3, similar to the gatekeeper F691L, and promotes resistance to gilteritinib. Our data fit nicely with recent data from a mutagenesis screen of Ba/F3 cells with FLT3-ITD that identified F691L in addition to D698N, G697S, and Y693C/N as mutations that drive resistance to gilteritinib[6]. Modeling of these mutations indicates that they cause the loss of hydrogen bonding that accommodates the FLT3 side chain, leading to a steric clash between the tetrahydropyran ring of gilteritinib and FLT3[6]. Given the proximity of N701K to these mutations, we speculate that

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130 the mechanism of resistance to gilteritinib imparted by this mutation is similar (**Supplemental**
131 **Figure 2**). Importantly, these complementary methods identify a common hotspot for gilteritinib
132 resistance mutations (**Supplemental Figure 3**). Given the increasing use of gilteritinib in the
133 clinic, we anticipate that additional resistance mutations will likely be identified in patients. Of note,
134 the N701K mutation appears to be more resistant to type I inhibitors but retains sensitivity to type
135 II inhibitors such as quizartinib (**Supplemental Figure 1**), implicating that TKI class switching
136 could serve as a promising avenue to mitigate development of gilteritinib resistance. The use of
137 type I FLT3 inhibitors following the acquisition of resistance to type II inhibitors is a well-
138 established approach to overcome resistance. However, what makes the case with the N701K
139 mutation interesting is acquired sensitivity to a type II inhibitor following development of resistance
140 to a type I inhibitor, which is a largely underappreciated concept. This knowledge can be used to
141 help rationally sequence FLT3 inhibitors upon development of resistance.
142

143 **FIGURE LEGENDS:**
144

145 **Figure 1: FLT3^{N701K} is an oncogenic mutation. A.** Model of early and late gilteritinib resistance.
146 MOLM14 (N = 8) and MV4;11 (N = 8) cultures were continuously treated with gilteritinib and
147 exogenous microenvironmental ligands (FGF2 or FL, 10 ng/mL) to recapitulate the role of the
148 marrow microenvironment in the development of early resistance. Following ligand withdrawal,
149 cultures become transiently sensitive to gilteritinib again, but eventually become resistant with the
150 outgrowth of NRAS and FLT3 resistance mutations that drive late resistance. **B.** Potential clonal
151 evolution paths resulting in the outgrowth of the FLT3^{N701K} mutation in three cultures. Mutations
152 were identified by whole exome sequencing and displayed via fishplots[14]. All mutations were
153 confirmed by Sanger sequencing. **C.** Gene schematic depicts location of the FLT3^{N701K} point
154 mutation relative to FLT3 gatekeeper (F691L) and activating loop (D835Y) mutations. The location
155 of the following domains is included: immunoglobulin (Ig)-like loops, transmembrane (TM),

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juxtamembrane (JM), and tyrosine kinase. **D.** Ribbon diagram mapping the location of FLT3^{N701K} onto the crystal structure of the FLT3 kinase domain. Diagram was adapted from PDB 1RJB[3] and visualized with the UCSF Chimera software[15]. **E.** FLT3^{N701K} transforms the murine Ba/F3 pro-B cell line and enables IL-3 independent growth. No growth was observed in parental Ba/F3 cells or cells harboring an empty vector (pMX-puro), wildtype (WT) FLT3, or FLT3^{F691L}. Total viable cells are plotted over time and cell growth was measured after the withdrawal of IL-3. This experiment was repeated at least twice with consistent results.

Figure 2: FLT3^{N701K} confers resistance to gilteritinib. A-B. Ba/F3 cells expressing FLT3^{N701K} and FLT3$^{ITD+N701K}$ demonstrate reduced gilteritinib sensitivity. FLT3^{D835Y} and FLT3$^{ITD+F691L}$ were used as historical controls. Six replicates of WT and mutant FLT3 Ba/F3 cells were plated with a dose gradient of gilteritinib (0 – 1000 nM) for 72 hrs. FLT3^{WT} cells were plated in media supplemented with IL-3. Cell viability was determined using a tetrazolamine-based viability assay. Viability is represented as a percentage of the untreated control. The average mean ± SEM is shown. **C.** Expression of total and phosphorylated FLT3 is increased in mutant-transformed Ba/F3 cells relative to cells harboring empty vector. All mutants phosphorylate canonical downstream effectors – STAT5, AKT, and ERK. GAPDH served as a loading control. Prior to lysis, empty vector cells were grown in IL-3 supplemented media and all lines were starved overnight in 0.1% BSA RPMI. **D.** FLT3 activity is sustained with mutations in N701K and F691L. Ba/F3 cells harboring FLT3^{ITD}, FLT3$^{ITD+F701K}$, and FLT3$^{ITD+F691L}$ were treated with gilteritinib (0 – 400 nM) for 90 minutes and lysed for immunoblot analysis[4, 6].

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AUTHOR CONTRIBUTIONS:

Study supervision: E. Traer

Conception and design: S.K. Joshi, E. Traer

Development of methodology: S.K. Joshi, E. Traer

Acquisition of data: S.K. Joshi, S. Sharzehi, J. Pittsenbarger

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.K. Joshi, S. Sharzehi, J. Pittsenbarger, D. Bottomly, C.E. Tognon, S.K. McWeeney, B.J. Druker, E. Traer

Writing, review, & editing of the manuscript: S.K. Joshi, J. Pittsenbarger, S. Sharzehi, D. Bottomly, C.E. Tognon, S.K. McWeeney, B.J. Druker, E. Traer

Development of prioritization framework to assist in rigor and reproducibility: D. Bottomly, S.K. McWeeney

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B.J.D. potential competing interests – SAB: Aileron Therapeutics, Therapy Architects (ALLCRON), Cepheid, Vivid Biosciences, Celgene, RUNX1 Research Program, Novartis, Gilead Sciences (inactive), Monojul (inactive); SAB & Stock: Aptose Biosciences, Blueprint Medicines,

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208 EnLiven Therapeutics, Iterion Therapeutics, Third Coast Therapeutics, GRAIL (SAB inactive);
209 Scientific Founder: MolecularMD (inactive, acquired by ICON); Board of Directors & Stock:
210 Amgen; Vincera Pharma; Board of Directors: Burroughs Wellcome Fund, CureOne; Joint Steering
211 Committee: Beat AML LLS; Founder: VB Therapeutics; Sponsored Research Agreement:
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219
220
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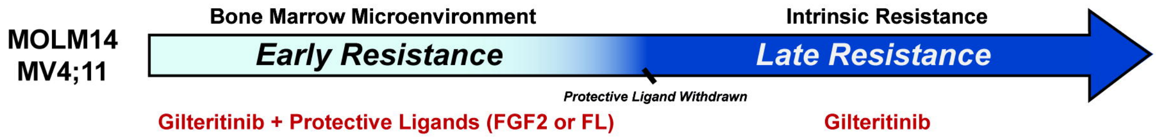
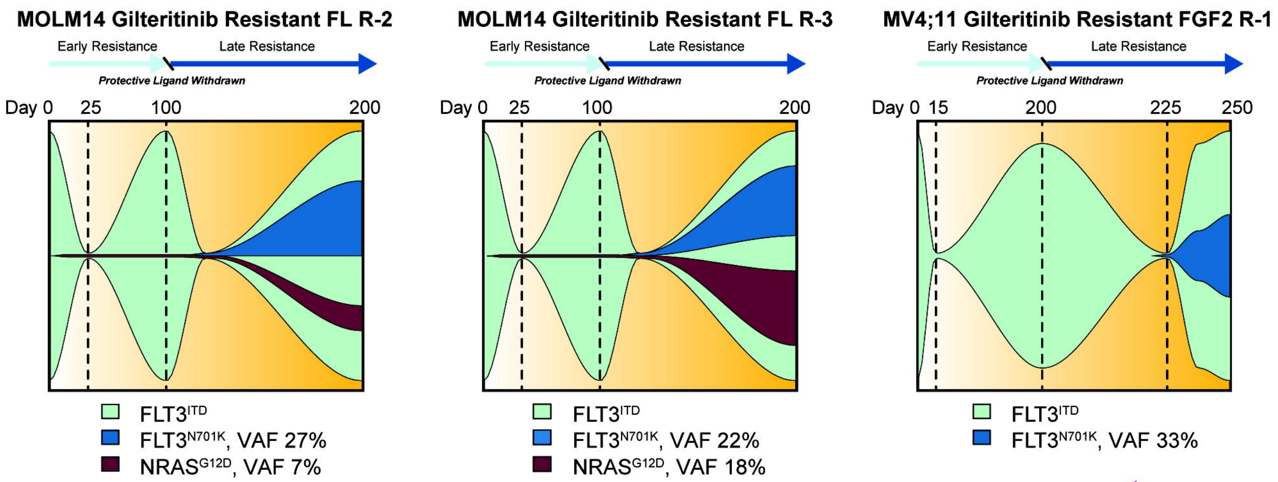
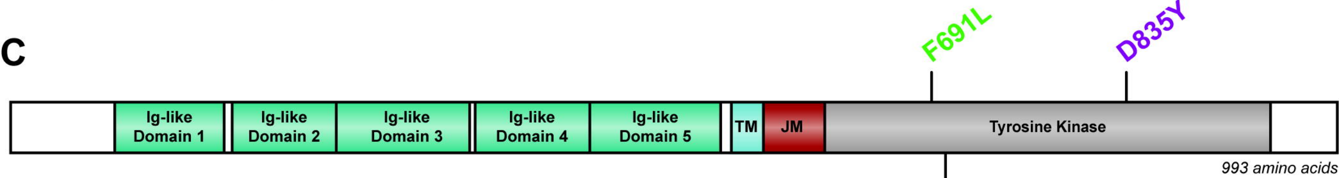
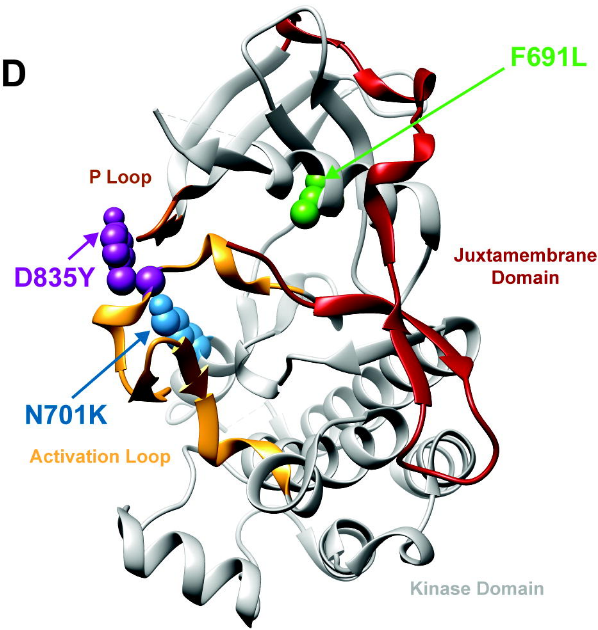
266

Figure 1









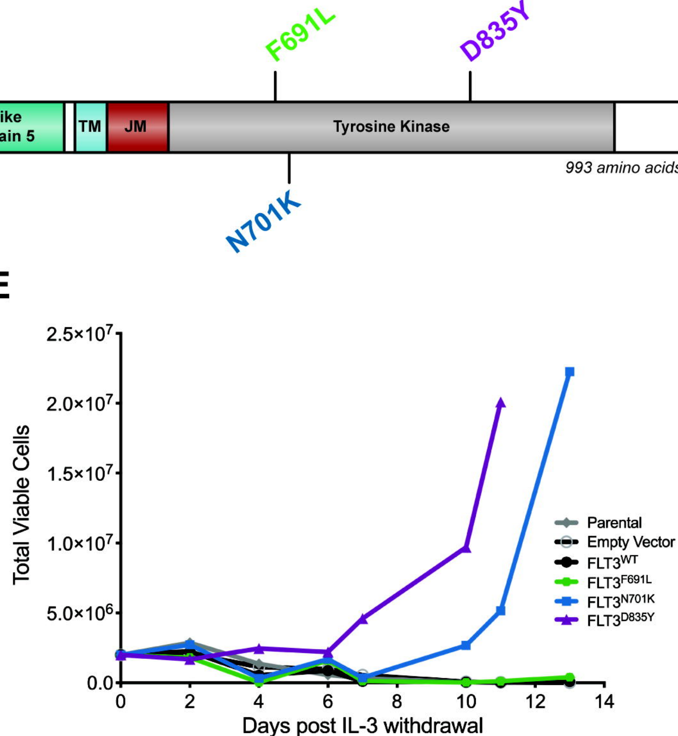


Figure 2

A

% Cell Viability

125
100
75
50
25

10
100
1000

Gilteritinib Concentration (nM)

FLT3^WT + IL-3
FLT3^N701K
FLT3^D835Y

B

% Cell Viability

125
100
75
50
25

10
100
1000

Gilteritinib Concentration (nM)

FLT3^WT + IL-3
FLT3^ITD
FLT3^ITD + F691L
FLT3^ITD + N701K

C

Empty Vector
FLT3^N701K
FLT3^ITD
FLT3^ITD + F691L
FLT3^ITD + N701K

pFLT3 (Y589/591)
Total FLT3
pSTAT5 (Y694)
Total STAT5
pAKT (S473)
Total AKT
pERK (T202/Y204)
Total ERK
GAPDH

D

FLT3^ITD

Gilteritinib (nM) 0 25 50 100 200 400
pFLT3 (Y589/591)
Total FLT3
GAPDH

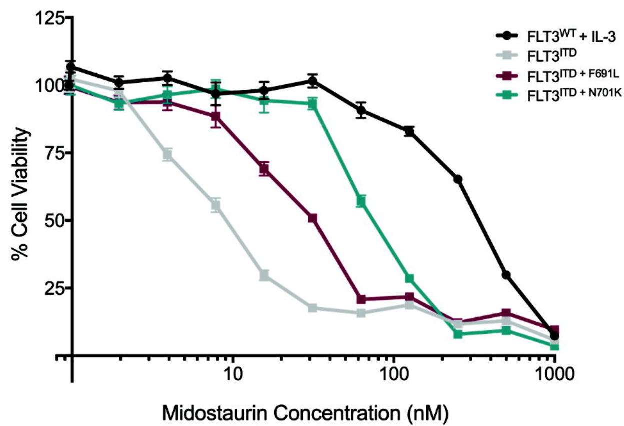
FLT3^ITD + N701K

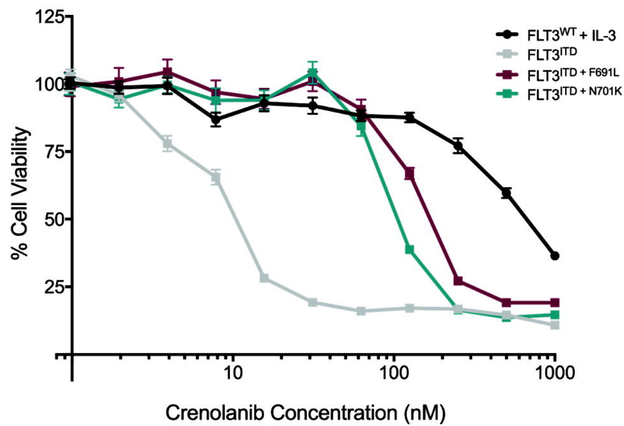
Gilteritinib (nM) 0 25 50 100 200 400
pFLT3 (Y589/591)
Total FLT3
GAPDH

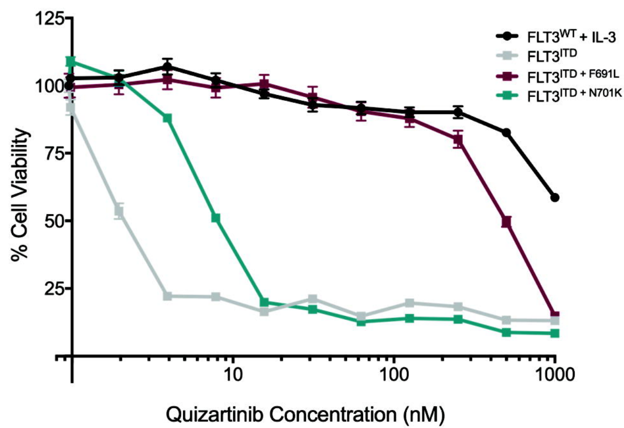
FLT3^ITD + F691L

Gilteritinib (nM) 0 25 50 100 200 400
pFLT3 (Y589/591)
Total FLT3
GAPDH

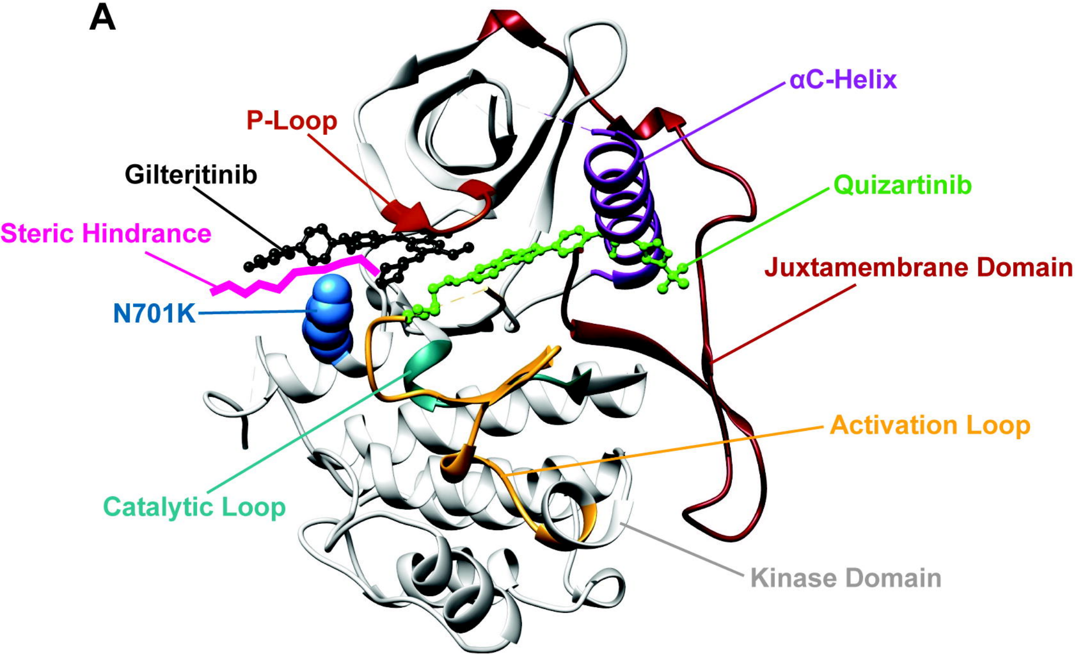
Supplemental Figure 1



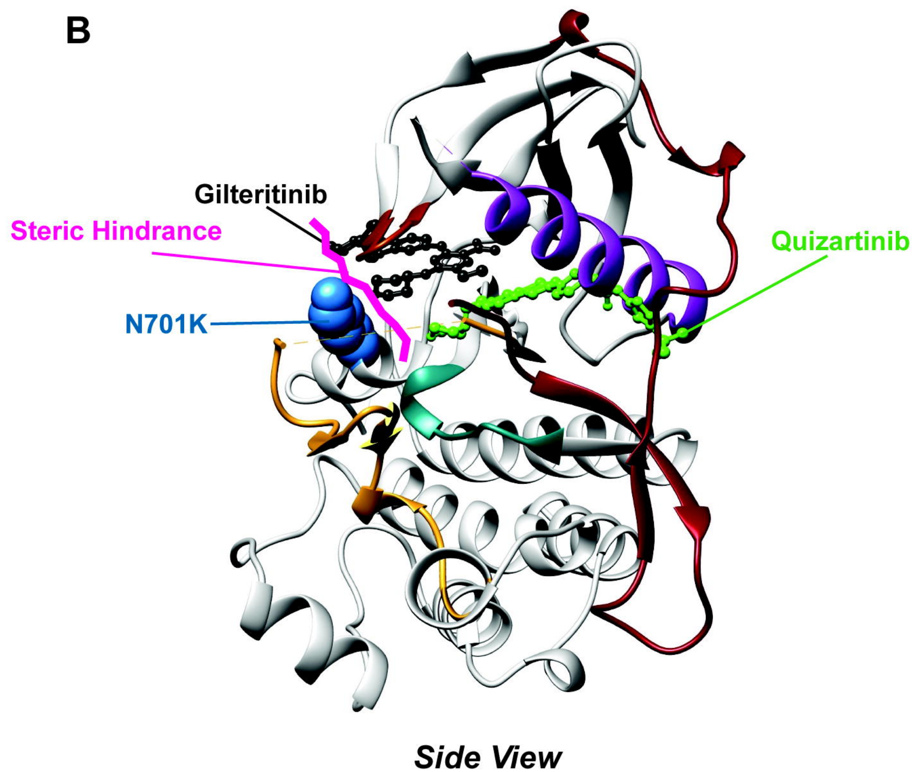




Supplemental Figure 2



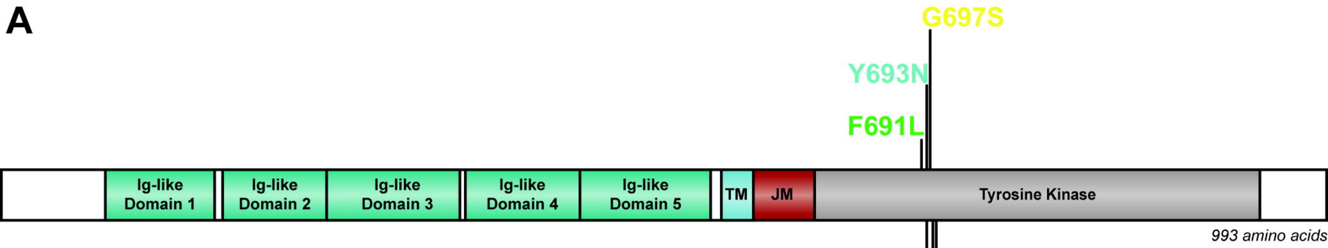
Front View



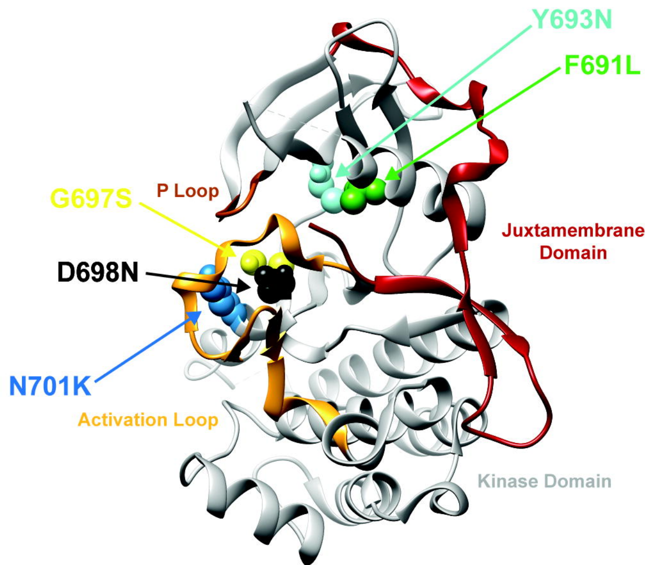
Side View

Supplemental Figure 3

A



B



C