

Table S1. Binding Affinity of Quizartinib to FLT3 D835 Mutant Isoforms

FLT3 Isoform	Kd (nM)
FLT3 (JM-minus)	1.8
FLT3 (JM-minus)/D835H	2.1
FLT3 (JM-minus)/D835V	4.8
FLT3 (JM-minus)/D835Y	7

Table S2. Inhibitory Concentration 50 (IC₅₀) of Quizartinib for Cell Lines Containing FLT3 D835 Mutant Isoforms

Cell Line	IC ₅₀ (nM)
Parental Ba/F3 + IL3	>1000nM
FLT3-ITD	0.25±0.23
D835V	37±12
D835Y	5.8±3.5
D835F	42±26
ITD + D835V	100±23
ITD + D835Y	24±3.6
ITD + D835F	124±37

Table S3. Frequency of D835 Mutations in ITD+ and Native *FLT3* Alleles in Pretreatment Samples

Subject Number	Sequences ITD+/Native <i>FLT3</i>	Mutation	Observed Alternative Codon Frequency (of ITD+ or Native <i>FLT3</i> alleles)
1005-006	ITD+	None	N/A
	Native <i>FLT3</i>	None	N/A
1005-007	ITD+	None	N/A
	Native <i>FLT3</i>	None	N/A
1005-009	ITD+	None	N/A
	Native <i>FLT3</i>	D835Y	10.40%
	Native <i>FLT3</i>	D835V	8.60%
	Native <i>FLT3</i>	D835F	3.70%
1009-003	ITD+	None	N/A
	Native <i>FLT3</i>	None	N/A
1009-007	ITD+	None	N/A
	Native <i>FLT3</i>	None	N/A
1011-006	ITD+	None	N/A
	Native <i>FLT3</i>	None	N/A
1011-007	ITD+	None	N/A

Table S4. Mutant Allele Frequencies in Patient Blasts after Relapse on Quizartinib

Patient	Genomic Allele Frequency in Bulk (Unsorted) Cells					Genomic Allele Frequency Based on Sorted Single Cell Genotyping					Expressed Allele Frequency in cDNA from Bulk (Unsorted) Cells				
	ITD	D835Y (TAC)	D835Y (TAT)	D835V (GTT)	D835F (TTT)	ITD	D835Y (TAC)	D835Y (TAT)	D835V (GTT)	D835F (TTT)	ITD	D835Y (TAC)	D835Y (TAT)	D835V (GTT)	D835F (TTT)
1005-007	51%		Too rare to quantitate	29%		48%		3%	32%		60%		2%	38%	
1005-009	89%	6%	42%			89%	2%	44%			98%		50%		
1009-003	52%		27%*	Too rare to quantitate	27%*	52%		14%	3%	15%	55%		8%	2%	8%

*percentage inclusive of both D835V (TAT) and D835F (TTT) mutation

Table S5. Genotype of Sorted Single Cells From Patients at Relapse on Quizartinib

Patient 1005-007	
Mutation Status	Number of Cells With Genotype
No ITD Mutation/No D835 Mutation	35
Heterozygous ITD/No D835 Mutation	102
Homozygous ITD/No D835 Mutation	25
No ITD Mutation/Heterozygous D835V (GTT)	7
Heterozygous ITD/Heterozygous D835V (GTT)	48
Homozygous ITD/Heterozygous D835V (GTT)	6
No ITD Mutation/Homozygous D835V (GTT)	11
Heterozygous ITD/Homozygous D835V (GTT)	50
Homozygous ITD/Homozygous D835V (GTT)	8
No ITD Mutation/Heterozygous D835Y (TAT)	2
Heterozygous ITD/Heterozygous D835Y (TAT)	13
Homozygous ITD/Heterozygous D835Y (TAT)	1
No ITD Mutation/Homozygous D835Y (TAT)	0
Heterozygous ITD/Homozygous D835Y (TAT)	2
Homozygous ITD/Homozygous D835Y (TAT)	0
Total Number of Cells Genotyped	310
Patient 1005-009	
Mutation Status	Number of Cells With Genotype
No ITD Mutation/No D835 Mutation	12
Heterozygous ITD/No D835 Mutation	10
Homozygous ITD/No D835 Mutation	85
No ITD Mutation/Heterozygous D835Y (TAT)	0
Heterozygous ITD/Heterozygous D835Y (TAT)	2
Homozygous ITD/Heterozygous D835Y (TAT)	71
No ITD Mutation/Homozygous D835Y (TAT)	0
Heterozygous ITD/Homozygous D835Y (TAT)	5
Homozygous ITD/Homozygous D835Y (TAT)	0
No ITD Mutation/Heterozygous D835Y (TAC)	0
Heterozygous ITD/Heterozygous D835Y (TAC)	1
Homozygous ITD/Heterozygous D835Y (TAC)	2
No ITD Mutation/Homozygous D835Y (TAC)	0
Heterozygous ITD/Homozygous D835Y (TAC)	0
Homozygous ITD/Homozygous D835Y (TAC)	0
Total Number of Cells Genotyped	188
Patient 1009-003	
Mutation Status	Number of Cells With Genotype
No ITD Mutation/No D835 Mutation	51
Heterozygous ITD/No D835 Mutation	45

Homozygous ITD/No D835 Mutation	38
No ITD Mutation/Heterozygous D835F (TTT)	1
Heterozygous ITD/Heterozygous D835F (TTT)	25
Homozygous ITD/Heterozygous D835F (TTT)	8
No ITD Mutation/Homozygous D835F (TTT)	3
Heterozygous ITD/Homozygous D835F (TTT)	8
Homozygous ITD/Homozygous D835F (TTT)	12
No ITD Mutation/Heterozygous D835Y (TAT)	4
Heterozygous ITD/Heterozygous D835Y (TAT)	29
Homozygous ITD/Heterozygous D835Y (TAT)	15
No ITD Mutation/Homozygous D835Y (TAT)	8
Heterozygous ITD/Homozygous D835Y (TAT)	6
Homozygous ITD/Homozygous D835Y (TAT)	1
No ITD Mutation/Heterozygous D835V (GTT)	0
Heterozygous ITD/Heterozygous D835V (GTT)	13
Homozygous ITD/Heterozygous D835V (GTT)	3
No ITD Mutation/Homozygous D835V (GTT)	0
Heterozygous ITD/Homozygous D835V (GTT)	1
Homozygous ITD/Homozygous D835V (GTT)	0
Total Number of Cells Genotyped	271

Table S6. Genotype of Colonies Grown from a Patient Relapsed on Quizartinib

Patient 3351-1085	DMSO	Quizartinib 20nM
No ITD Mutation/No D835 Mutation	0	4
Heterozygous ITD/No D835 Mutation	9	8
Homozygous ITD/No D835 Mutation	0	0
No ITD Mutation/Heterozygous D835Y (TAT)	2	6
Heterozygous ITD/Heterozygous D835Y (TAT)	71	53
Homozygous ITD/Heterozygous D835Y (TAT)	1	3
No ITD Mutation/Homozygous D835Y (TAT)	0	2
Heterozygous ITD/Homozygous D835Y (TAT)	0	3
Homozygous ITD/Homozygous D835Y (TAT)	0	2
Total Number of Colonies with Genotype	83	81

Table S7. List of 33 Hematologic-Malignancy Associated Genes Sequenced by Targeted Exome Sequencing of Mutation Hotspots

NPM1
TP53
WT1
PHF6
DNMT3A
TET2
IDH1
IDH2
FLT3
PTPN11
JAK2
KIT
NRAS
KRAS
CBL
GNAS
PTEN
BRAF
ASXL1
EZH2
SF3B1
RUNX1
ETV6
ATM
CDKN2A
DDX3X
FBXW7
KLHL6
MAPK1
MYD88
NOTCH1
XPO1
ZMYM3

Table S8. Hematologic-Malignancy Associated Mutations Identified in Pre-Treatment Samples by Exome Sequencing of Mutation Hotspots

Patient ID	Gene	Protein Change	Nucleotide change	Abnormal Reads	Read Depth	Variant Allele Frequency (%)
1009-003	DNMT3A	p.R882H	c.G2645A	253	573	44.15
1009-003	RUNX1	p.T257fs	c.769_791delinsGG CCTCT	104	252	41.26
1009-003	FLT3	p.D593delinsELPSY EYFYVD	c.1779_1780insGTT ACCCTCGTATGA GTACTTCTACGT TGA	894	1983	45.08
3351-1085	NPM1	p.W288fs	c.863_864insTCTG	149	715	20.84
3351-1085	DNMT3A	p.R882H	c.G2645A	262	1090	24.04
3351-1085	FLT3	p.N587_E588ins?	c.1760_1761insCTT CTACGTTGATTT CAGAGAATATGA ATATGATCTCAA ATGGGAGTTTCC AAGAGAAAATTT AGAGTTTGAGGC GA	458	1621	28.25
1005-007	NPM1	p.W288fs	c.863_864insTCTG		186	33.87
1005-007	TET2	p.Q1327P	c.A3980C	1514	13051	11.6
1005-007	TET2	p.?	c.3595-1A	2168	5166	41.97
1005-007	FLT3	p.D600delinsFREY EYD	c.1798_1799insTTC AGAGAATATGAA TAT	342	1712	20
1005-007	FLT3	p.K602delinsEY EY DLK	c.1804_1805insGA ATATGAATATGA TCTC	75	1712	4.38
1005-009	FLT3	pM578_V579ins?	c.1735_1736insAGT ACTTCTACGTTG ATTTTCAGAGAAT ATGAATATGATC TCAAATGGGAG	484	1361	35.56

Table S9. Off-Target Mutations Acquired In Resistance Samples										
Patient ID	Gene	Symbol	Entrez Gene Name	Location	Type(s)	Variant Allele Frequency	Protein Change	Nucleotide Change	Variant Classification	Variant Type
1005-007	ZNF665	ZNF665	zinc finger protein 665	Nucleus	other	0.07	p.Asp97Asn	c.289G>A	missense_variant	SNP
1005-007	SRSF6	SRSF6	serine/arginine-rich splicing factor 6	Nucleus	other	0.11	p.Arg321Thr	c.962G>C	missense_variant	SNP
1005-007	KIF3C	KIF3C	kinesin family member 3C	Cytoplasm	other	0.10	p.Arg45Trp	c.133C>T	missense_variant	SNP
1009-003	LPHN2	LPHN2	latrophilin 2	Plasma Membrane	G-protein coupled receptor	0.19	p.Glu242Lys	c.724G>A	missense_variant	SNP
1009-003	ALPP	ALPP	alkaline phosphatase, placental	Plasma Membrane	phosphatase	0.14	p.Leu13del	c.37_39delCTG	inframe_deletion	DEL
1009-003	HCRT	HCRT	hypocretin (orexin) neuropeptide precursor	Extracellular Space	other	0.09	p.Leu22del	c.65_67delTGC	disruptive_inframe_deletion	DEL
1009-003	DERL3	DERL3	derlin 3	Cytoplasm	other	0.07		c.*314_*739delAAGAAATTG AACATGACC TGTTG	splice_region_variant	DEL
1009-003	OR1N2	OR1N2	olfactory receptor, family 1, subfamily N, member 2	Plasma Membrane	G-protein coupled receptor	0.07	p.Ala184fs	c.550dupG	frameshift_variant	INS
1009-003	FFAR2	FFAR2	free fatty acid receptor 2	Plasma Membrane	G-protein coupled receptor	0.06		c.172_174delCTG	sequence_feature	DEL
1009-003	INPP5D	INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	Cytoplasm	phosphatase	0.04	p.Gln303*	c.907C>T	stop_gained	SNP
3351-1085	TNF	TNF	tumor necrosis factor	Extracellular Space	cytokine	0.47	p.Ile159fs	c.475dupA	frameshift variant	INS
3351-1085	MRE11A	MRE11A	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	Nucleus	enzyme	0.42	p.Ile245Met	c.735C>G	missense variant	SNP
3351-1085	GPR50	GPR50	G protein-coupled receptor 50	Plasma Membrane	G-protein coupled	0.39	p.Asn298Ile	c.893A>T	missense variant	SNP

					receptor					
3351-1085	ATAD3B	ATAD3B	ATPase family, AAA domain containing 3B	Nucleus	other	0.09	p.Met619Val	c.1855A>G	missense variant	SNP
3351-1085	ZIC3	ZIC3	Zic family member 3	Nucleus	transcription regulator	0.09		c.135_137delCGC	sequence feature	DEL
3351-1085	APOA5	APOA5	apolipoprotein A-V	Extracellular Space	transporter	0.05	p.Ala219fs	c.654dupC	frameshift variant	INS

Table S10. Exome Sequencing Report						
	1009-003 Baseline	1009-003 Relapse	3351-1085 Baseline	3351-1085 Progression	1005-007 Baseline	1005-007 Progression
total	119580342	107451591	113551820	106921756	105485501	110303560
duplicates	13609080	12266016	13163160	19031431	13894638	14742269
mapped	119212640	107136201	113141045	106360993	105161220	109969986
paired_in_sequence	119580342	107451591	113551820	106921756	105485501	110303560
read1	59790183	53725805	56775915	53460892	52742752	55151781
read2	59790159	53725786	56775905	53460864	52742749	55151779
properly_paired	118443620	106460016	112260858	105089702	104644412	109269006
both_mapped	118844938	106820811	112730270	105800230	104836939	109636412
singletons	367702	315390	410775	560763	324281	333574
mate_mapped_chr	300798	272463	352572	561966	92835	269694
mate_mapped_chr_highq	281045	255748	329980	524338	82743	251905
bait_set	SureSelect_Human_All_Exon_V5	SureSelect_Human_All_Exon_V5	SureSelect_Human_All_Exon_V5	SureSelect_Human_All_Exon_V5	SureSelect_Human_All_Exon_V5	SureSelect_Human_All_Exon_V5
genome_size	3095693983	3095693983	3095693983	3095693983	3095693983	3095693983
bait_territory	50621019	50621019	50621019	50621019	50621019	50621019
target_territory	50621019	50621019	50621019	50621019	50621019	50621019
bait_design_efficiency	1	1	1	1	1	1
total_reads	119580342	107451591	113551820	106921756	105485501	110303560
pf_reads	119580342	107451591	113551820	106921756	105485501	110303560
pf_unique_reads	105971262	95185575	100388660	87890325	91590863	95561291
pct_pf_reads	1	1	1	1	1	1
pct_pf_uq_reads	0.886193	0.885846	0.884078	0.822006	0.868279	0.866348
pf_uq_reads_aligned	103866987	93327144	98291002	85827697	89703285	93608648
pct_pf_uq_reads_aligned	0.980143	0.980476	0.979105	0.976532	0.979391	0.979567
pf_uq_bases_aligned	10336918469	9288435316	9778681589	8535750070	8927848197	9317717344
on_bait_bases	6332047586	5741129982	6096493153	5391062709	5514467991	5845954799
near_bait_bases	2166968268	1946401973	1962563246	1683960135	1844233287	1941846891
off_bait_bases	1837902615	1600903361	1719625190	1460727226	1569146919	1529915654
on_target_bases	6332047586	5741129982	6096493153	5391062709	5514467991	5845954799
pct_selected_bases	0.8222	0.827646	0.824145	0.828869	0.824241	0.835806
pct_off_bait	0.1778	0.172354	0.175855	0.171131	0.175759	0.164194
on_bait_vs_selected	0.745033	0.746811	0.756477	0.761985	0.749381	0.750655

mean_bait_coverage	125.08732	113.413955	120.434027	106.498502	108.936329	115.484732
mean_target_coverage	125.948337	114.198045	121.29554	107.274637	109.673766	116.279304
pct_usable_bases_on_bait	0.529522	0.534299	0.536891	0.504206	0.52277	0.529988
pct_usable_bases_on_target	0.529522	0.534299	0.536891	0.504206	0.52277	0.529988
fold_enrichment	37.461074	37.799143	38.126499	38.62423	37.773216	38.368345
zero_cvg_targets_pct	0.00937	0.009335	0.009713	0.009904	0.009253	0.00937
fold_80_base_penalty	2.099139	2.076328	2.021592	2.02405	1.95846	2.004816
pct_target_bases_2x	0.992111	0.99204	0.991837	0.991548	0.99217	0.992105
pct_target_bases_10x	0.986284	0.985521	0.986314	0.984442	0.986389	0.986216
pct_target_bases_20x	0.973169	0.969706	0.973502	0.966647	0.972177	0.972399
pct_target_bases_30x	0.948944	0.939618	0.949511	0.933131	0.944543	0.946293
pct_target_bases_40x	0.910945	0.893028	0.911509	0.881896	0.899928	0.904975
pct_target_bases_50x	0.860021	0.832737	0.860016	0.816553	0.840265	0.849268
pct_target_bases_100x	0.544693	0.488868	0.535713	0.456617	0.484915	0.511791
hs_library_size	197725636	178233033	183751119	107504064	141605166	155580831
hs_penalty_10x	3.494423	3.427103	3.305254	3.308846	3.250826	3.266524
hs_penalty_20x	3.561838	3.502856	3.36919	3.428821	3.330704	3.344205
hs_penalty_30x	3.632997	3.575243	3.44023	3.552129	3.420568	3.430518
hs_penalty_40x	3.707902	3.659413	3.513046	3.698766	3.515424	3.515104
hs_penalty_50x	3.784305	3.750317	3.590835	3.858733	3.616271	3.611428
hs_penalty_100x	4.269688	4.291702	4.0597	5.082483	4.303227	4.216305
at_dropout	0	0	0	0	0	0
gc_dropout	0	0	0	0	0	0
sample						
library						
read_group						
median_insert_size	197	197	192	188	195	196
mean_insert_size	208.676651	208.624277	202.321265	198.263534	206.639724	207.107335
mean_coverage	126.55	114.74	121.87	107.77	110.22	116.86

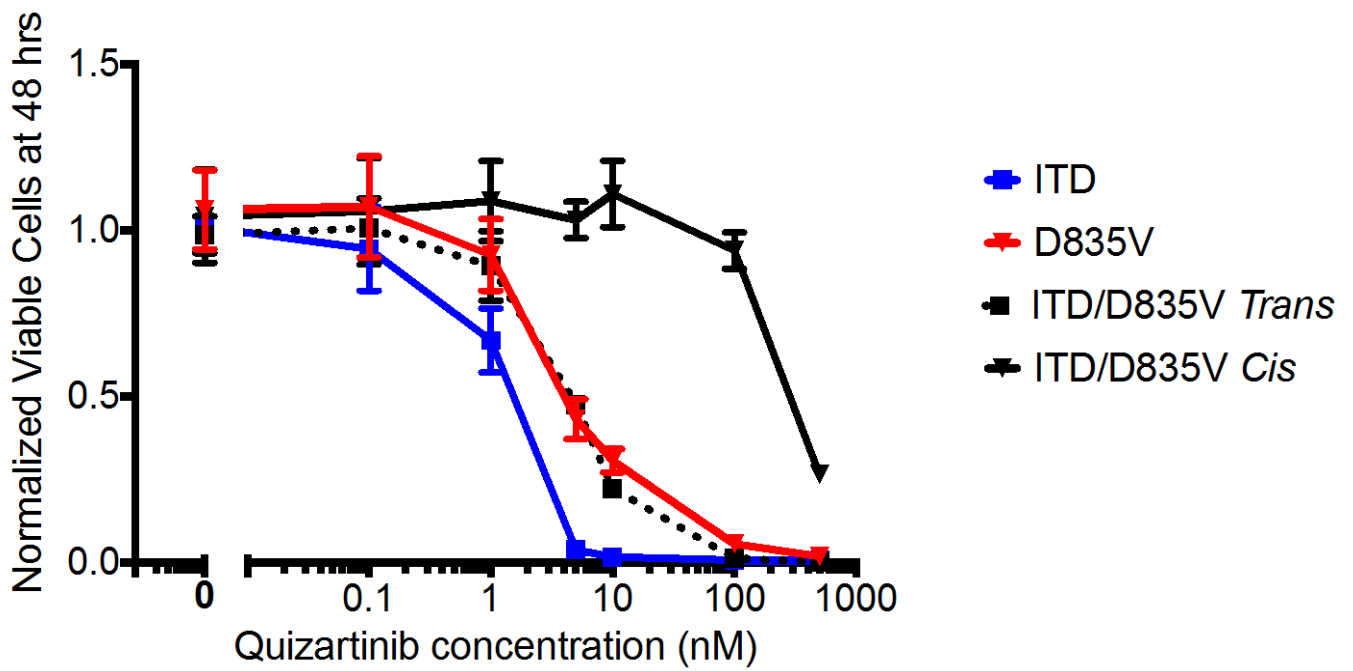


Figure S1. *FLT3* ITD and D835 Mutations in *Cis* and in *Trans* Confer Relative Resistance to Quizartinib.

Normalized cell viability of Ba/F3 populations stably expressing *FLT3* mutant isoforms after 48 hours in various concentrations of quizartinib (error bars represent s.d. of triplicates from the same experiment). Data shown is representative of biological triplicate experiments.

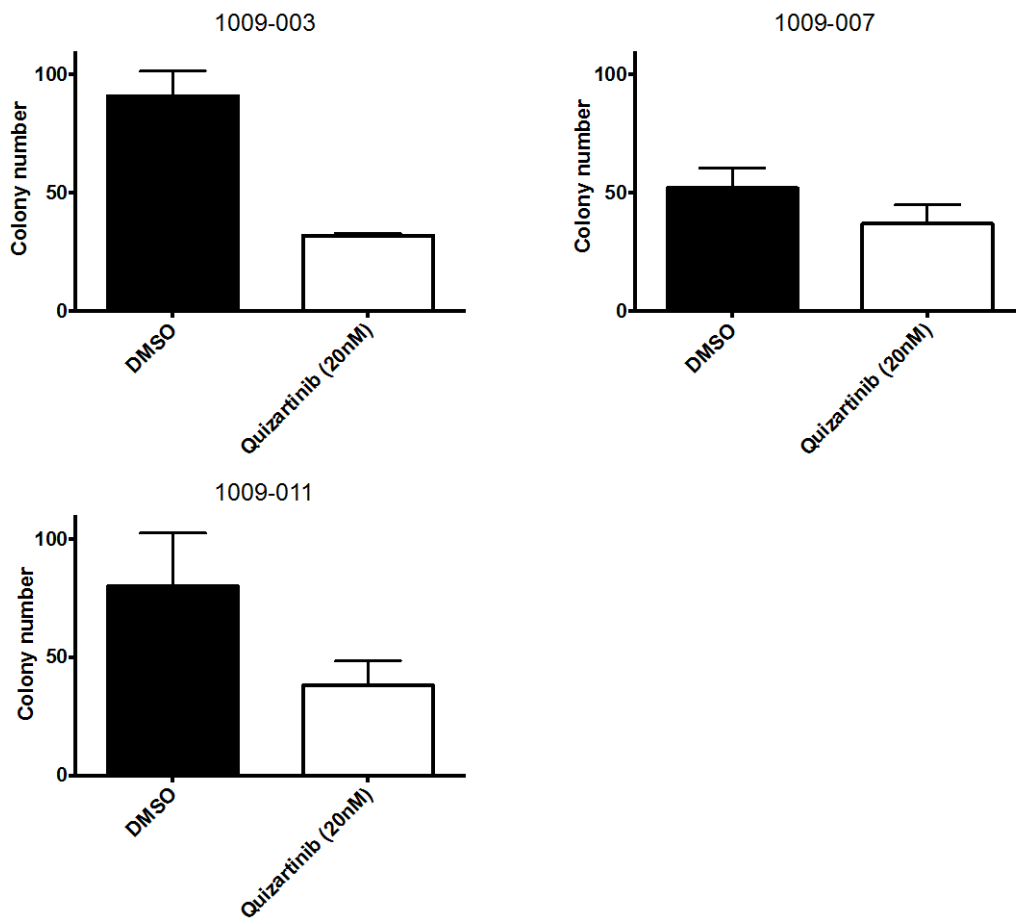


Figure S2. Quizartinib Suppresses Colony Formation in Pre-Treatment Samples From Patients Who Experience Clinical Response to Quizartinib. Frozen pre-treatment blood or bone marrow mononuclear cells are thawed and grown in triplicate in methylcellulose with DMSO or 20nM quizartinib. Colonies are counted after 14 days.