Supplemental Methods

Generation of Molm14 FLT3 mutant cell lines. The *FLT3*-ITD-positive parental Molm14 cells were a gift from Dr. Scott Kogan in 2008. Cell lines resistant to quizartinib were generated either by culturing parental Molm14 cells in liquid RPMI media containing escalating doses of quizartinib (0.5nM to 20nM) or by plating cells in RPMI media with 20% FCS in soft agar in the presence of 20nM quizartinib. Resistant cells generated in liquid media were sub-cloned prior to Sanger sequencing. Resistant cells generated in soft agar were expanded in liquid media supplemented with 20nM quizartinib before sequencing. In each case, the FLT3 KD was sequenced to confirm acquisition of indicated FLT3 mutations.

Molm14 Cell-Viability Assay. Molm14 parental and FLT3 mutant cells were plated in 0.1 to 1000nM quizartinib or gilteritinib in triplicate and expanded for 2 days. Viability was assessed and IC50 values were calculated as previously described[12].

Table S1: Summary of FLT3 Mutation IC50 Values in Ba/F3 Cells					
Mutation	Mean IC50 (nM)	Std Dev IC50 (nM)	N		Mutation Location (in native FLT3, ITD, or ITD D835V background as indicated)
ITD	1.81	0.63	30		TKD Mutations (activation loop)
D835F	0.98	0.03	3		TKD Mutations (not in activation loop)
D835V	0.67	0.16	3		EC Domain Mutation (crenolanib-resistant)
D835Y	0.68	0.07	3		
D839G	0.99	0.09	3		
I836del	1.65	0.37	3		
N676K	0.65	0.17	3		
N841I	1.55	0.17	3		
N841T	1.54	0.13	3		
N841Y	0.93	0.05	3		
R845S	1.05	0.33	3		
Y842C	1.72	0.31	3		
ITD A848P	2.33	0.30	3		
ITD D698N	31.54	5.72	3		
ITD D835A	1.32	0.50	3		
ITD D835del	4.20	2.32	3		
ITD D835E	1.37	0.35	3		
ITD D835F	1.24	0.36	3		
ITD D835G	2.29	1.46	3		
ITD D835H	1.06	0.42	3		
ITD D835I	0.94	0.44	3		
ITD D835N	0.88	0.29	3		
ITD D835V	3.53	1.24	30		
ITD D835V D698N	31.10	0.43	3		
ITD D835V E778K	3.42	0.52	3		
ITD D835V E786K	1.82	0.35	3		
ITD D835V F691L	17.79	1.91	3		
ITD D835V G631R	5.51	0.59	3		
ITD D835V G669R	10.49	0.46	3		
ITD D835V G697S	99.86	14.71	3		
ITD D835V G822E	2.36	0.74	3		
ITD D835V G846D	4.65	1.32	3		
ITD D835V G846S	6.97	0.99	3		
ITD D835V M664I	2.48	0.94	3		
ITD D835V M855T	9.52	0.87	3		
ITD D835V S705N	2.49	0.26	3		
ITD D835V Y693C	34.18	3.87	3		
ITD D835V Y693N	32.96	3.65	3		

ITD D835Y	1.13	0.29	3		
ITD D839A	3.48	1.62	3		
ITD D839G	2.47	0.12	3		
ITD D839H	3.38	0.48	3		
ITD D839N	2.90	1.31	3		
ITD E778K	3.88	0.51	3		
ITD E786K	2.16	0.41	3		
ITD F691L	25.35	4.74	24		
ITD G631R	4.02	0.64	3		
ITD G669R	6.62	1.19	3		
ITD G697S	117.80	6.10	3		
ITD G757E	1.62	0.23	3		
ITD G822E	2.83	0.52	3		
ITD G846D	8.91	2.11	3		
ITD G846R	1.80	1.18	3		
ITD G846S	8.40	2.17	3		
ITD H721Y	1.81	0.33	3		
ITD I836del	5.39	1.75	3		
ITD K429E	6.02	0.67	3		
ITD M664I	2.19	1.00	3		
ITD M837I	1.60	0.56	3		
ITD M837K	2.04	0.49	3		
ITD M855T	7.11	1.54	3		
ITD N609T	3.52	0.72	3		
ITD N676K	3.57	1.06	3		
ITD N841H	1.84	0.74	3		
ITD N841I	4.94	0.45	3		
ITD N841K	1.30	0.20	3		
ITD N841T	3.47	0.92	3		
ITD N841Y	5.23	0.74	3		
ITD R834Q	3.77	2.31	3		
ITD R845G	3.65	2.33	3		
ITD S705N	2.24	0.49	3		
ITD Y693C	27.90	4.96	3		
ITD Y693N	23.76	4.11	3		
ITD Y842C	3.77	1.88	3		
ITD Y842H	3.03	0.90	3		

Table S2. Summary of FLT3 Inhibitor Mutagenesis Studies

Drug	Type 1 or Type 2 Inhibitor	FLT3 Mutation Background	Colonies Screened	# Unique Resistant Mutations Identified	# Predicted Mutations Acquired in Patients at Relapse	Reference
Quizartinib	Type 2	ITD	97	5	3	Smith, et. al, Nature 2012
PLX3397 (Pexadartinib)	Type 2	ITD	78	18	4*	Smith et. al, Cancer Discovery 2015
		ITD/F691L	117			
Ponatinib	Type 2	ITD	50	6	N.D.	Smith et. al, Blood 2013
Crenolanib	Type 1	ITD	N.S.	3	1	Smith et. al, PNAS 2014
		ITD/D835V	N.S.			
Gilteritinib	Type 1	ITD	64	5	1	Current
		ITD/D835V	72			

N.S. = not stated. N.D. = not done. *4 additional mutations occurred at amino acid sites predicted in mutagenesis screen.

Table S3. Screening and End of Treatment FLT3 KD Mutations in Patients Relapsed on Gilteritinib

Subject No.	Initial Dose	Age/ Sex/ Race[1]/ Weight	CR/CRh[2]	Best Overall Response[2]	Cytogenetic Risk Status	Timepoint	Specimen Type	Blast (%)	Mutation	VAF (%)
63	200 mg/day	70 Years/|Female/|W/|79.2 kg	Yes	CR	Intermediate: normal	SCREENING	BONE MARROW	95	ITD	38.59
						CYCLE 10 DAY 1|(Relapse)	BLOOD	7		ND
70	200 mg/day	70 Years/|Female/|W/|91.9 kg	Yes	CR	Intermediate: normal	SCREENING	BONE MARROW	88	ITD	1.24
									ITD	95.07
						EOT|(Relapse)	BONE MARROW		ITD	42.94
97	200 mg/day	54 Years/|Male/|W/|108.7 kg	Yes	CR	Intermediate: normal	SCREENING	BONE MARROW	33	ITD	20.77
						EOT|(Relapse)	BONE MARROW		ITD	7.19
146	120 mg/day	57 Years/|Female/|B/|54.8 kg	Yes	CR		SCREENING	BONE MARROW	3	ITD	2.01
						CYCLE 17 DAY 1|(Relapse)	BONE MARROW	3	ITD	1.58
201	120 mg/day	82 Years/|Female/|W/|95.3 kg	Yes	CR		SCREENING	BLOOD	14	ITD	7.99
									ITD	28.21
									p.F349L	49.67
						EOT|(Relapse)	BLOOD	29	ITD	8.78
									p.F349L	48.05
86	200 mg/day	24 Years/|Female/|W/|54.2 kg	No	CRp	Intermediate: normal	SCREENING	BONE MARROW	70	ITD	90.15
						CYCLE 7 DAY 1|(Relapse)	BONE MARROW		ITD	94.84

96	120 mg/day	27 Years/|Female/|W/|48.5 kg	No	CRp	Intermediate: normal	SCREENING	BONE MARROW	79	ITD	47.18
						CYCLE 7 DAY 1|(Relapse)	BONE MARROW	78	ITD	45.87
124	200 mg/day	61 Years/|Female/|W/|53.7 kg	Yes	CRp	Intermediate: normal	SCREENING	BONE MARROW	33	ITD	73.98
						EOT|(Relapse)	BONE MARROW	7	ITD	66.05
12	120 mg/day	76 Years/|Female/|W/|70.9 kg	No	CRi	Intermediate: normal	SCREENING	BONE MARROW	68	ITD	77.62
						EOT|(Relapse)	BLOOD	56		ND
24	200 mg/day	59 Years/|Female/|W/|60.3 kg	No	CRi		SCREENING	BONE MARROW	88	ITD	84.18
						CYCLE 3 DAY 1|(Relapse)	BONE MARROW	2	ITD	60.72
25	200 mg/day	39 Years/|Male/|W/|67.3 kg	No	CRi		SCREENING	BONE MARROW	96	ITD	98.04
									p.D835Y	25.11
						CYCLE 3 DAY 1|(Relapse)	BONE MARROW	6	ITD	88.79
									p.D835H	22.85
44	80 mg/day	51 Years/|Male/|W/|90.9 kg	No	CRi	Intermediate: normal	SCREENING	BONE MARROW	81	ITD	63.41
						EOT|(Relapse)	BLOOD	30	ITD	54.91
									p.N609T	0.86
62	200 mg/day	22 Years/|Female/|W/|53.6 kg	No	CRi	Intermediate: normal	SCREENING	BLOOD	79	ITD	41.03
									p.D835Y	16.60
						CYCLE 6 DAY 1|(Relapse)	BLOOD	1	ITD	0.87
106	120 mg/day	69 Years/|Male/|W/|87.1 kg	No	CRi	Intermediate: normal	SCREENING	BONE MARROW	78	ITD	79.96
						EOT|(Relapse)	BONE MARROW	9	ITD	43.84

121	80 mg/day	67 Years/|Male/|W/|91.6 kg	No	CRi	Intermediate: normal	SCREENING	BONE MARROW	2	ITD	26.77
									p.D835Y	3.33
						EOT|(Relapse)	BLOOD	4	ITD	29.60
									p.F691L	5.39
									p.D835Y	7.85
125	120 mg/day	71 Years/|Female/|W/|71.2 kg	Yes	CRi	Intermediate: normal	SCREENING	BONE MARROW	97	ITD	97.45
						EOT|(Relapse)	BLOOD	18	ITD	98.99
138	120 mg/day	84 Years/|Male/|W/|83.9 kg	Yes	CRi	Intermediate: +8	SCREENING	BONE MARROW	56	ITD	65.30
									p.D835H	18.51
						CYCLE 8 DAY 1|(Relapse)	BLOOD	14	ITD	40.60
									p.F691L	2.16
									p.F691L	4.90
142	120 mg/day	30 Years/|Female/|W/|124.1 kg	Yes	CRi	Favorable: inv(16)	SCREENING	BONE MARROW	58	ITD	48.71
						EOT|(Relapse)	BLOOD	22	ITD	28.14
									p.F691L	4.09
178	200 mg/day	68 Years/|Male/|W/|106.2 kg	No	CRi		SCREENING	BLOOD	82	ITD	37.95
									p.D835H	0.87
						EOT|(Relapse)	BONE MARROW	3	ITD	16.62
									p.D835H	10.88
209	120 mg/day	52 Years/|Female/|W/|56.7 kg	Yes	CRi	Intermediate: normal	SCREENING	BONE MARROW	2	ITD	10.90
									p.D835Y	0.67
						EOT|(Relapse)	BLOOD	8		ND
227	200 mg/day	60 Years/|Female/|W/|55.2 kg	No	CRi	Favorable: inv(16)	SCREENING	BONE MARROW	95	p.D835Y	40.42
						EOT|(Relapse)	BONE MARROW	67		ND
58	120 mg/day	78 Years/|Female/|W/|65.9 kg	No	PR	Intermediate: normal	SCREENING	BONE MARROW	61	ITD	35.44

						CYCLE 3 DAY 1|(Relapse)	BONE MARROW	18	ITD	46.05
21	120 mg/day	52 Years/|Female/|W/|46.3 kg	No	NR		SCREENING	BONE MARROW	90	ITD	80.64
						EOT|(Relapse)	BONE MARROW	23	p.D835Y	5.01
51	40 mg/day	73 Years/|Male/|W/|88.6 kg	No	NR	Unfavorable: complex	SCREENING	BONE MARROW	88	ITD	48.44
									p.D835Y	2.98
						EOT|(Relapse)	BONE MARROW	68	ITD	53.12
									p.F691L	7.31
									p.F691L	10.70
72	200 mg/day	67 Years/|Female/|W/|45.8 kg	No	NR	Unfavorable: complex	SCREENING	BONE MARROW	88	ITD	38.15
						CYCLE 5 DAY 1|(Relapse)	BONE MARROW	22		ND
87	120 mg/day	69 Years/|Male/|W/|92.1 kg	No	NR	Intermediate: normal	SCREENING	BONE MARROW	89	ITD	0.71
									ITD	88.62
						EOT|(Relapse)	BLOOD	33	ITD	90.83
118	120 mg/day	68 Years/|Female/|W/|84.5 kg	No	NR	Intermediate: normal	SCREENING	BONE MARROW	91	ITD	47.66
						EOT|(Relapse)	BONE MARROW	73		ND
186	200 mg/day	45 Years/|Female/|W/|59.5 kg	No	NR	Intermediate: normal	SCREENING	BONE MARROW		ITD	6.41
									p.D835H	1.01
						EOT|(Relapse)	BLOOD	19		ND
220	120 mg/day	59 Years/|Male/|W/|77.0 kg	No	NR	Intermediate: normal	SCREENING	BONE MARROW	22	ITD	10.43
						CYCLE 4 DAY 1|(Relapse)	BONE MARROW	27		ND
243	200 mg/day	79 Years/|Male/|W/|75.0 kg	No	NR	Unfavorable: del7q	SCREENING	BONE MARROW	91	ITD	21.50
									ITD	69.29
						EOT|(Relapse)	BLOOD	51	ITD	30.37

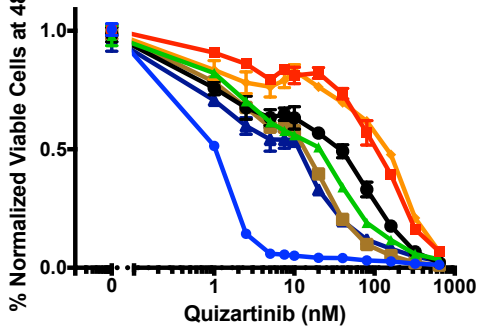
									ITD	49.01
									ITD	0.72
248	200 mg/day	35 Years/|Female/|W/|63.0 kg	No	NR	Unfavorable: complex	SCREENING	BLOOD	1	ITD	21.62
									p.D835Y	4.39
						CYCLE 2 DAY 1|(Relapse)	BLOOD	15	ITD	15.15
									p.D835Y	1.65

[1]Race: W-White.

[2]CR: complete remission, CRp: complete remission with incomplete platelet recovery, CRi: complete remission with incomplete hematological recovery, CRh: complete remission with partial hematological recovery, PR: partial response, NR: no response, NE: not evaluable.

EOT: end of treatment, VAF: variant frequency

A



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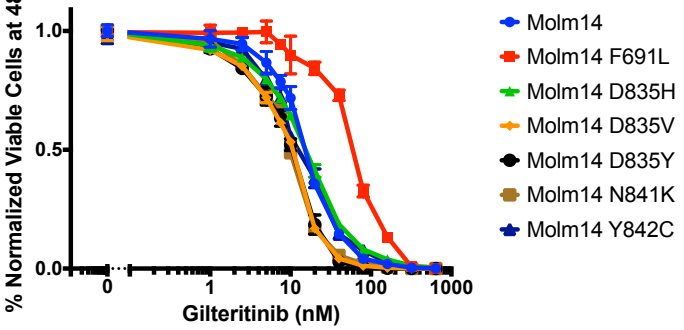

C

	Quizartinib		Gilteritinib	
Cell Line	IC50 (nM)	Std. Dev.	IC50 (nM)	Std. Dev.
Molm14 Parental	0.53	0.03	13.39	0.29
Molm14 D835H	19.8	0.66	14.16	0.53
Molm14 D835V	144.13	11.98	8.89	1.04
Molm14 D835Y	49.75	6.7	8.49	0.84
Molm14 F691L	122.87	26.07	42.16	6.11
Molm14 N841K	12.88	1.06	8.04	0.77
Molm14 Y842C	10.94	1.29	12.18	1.22

D

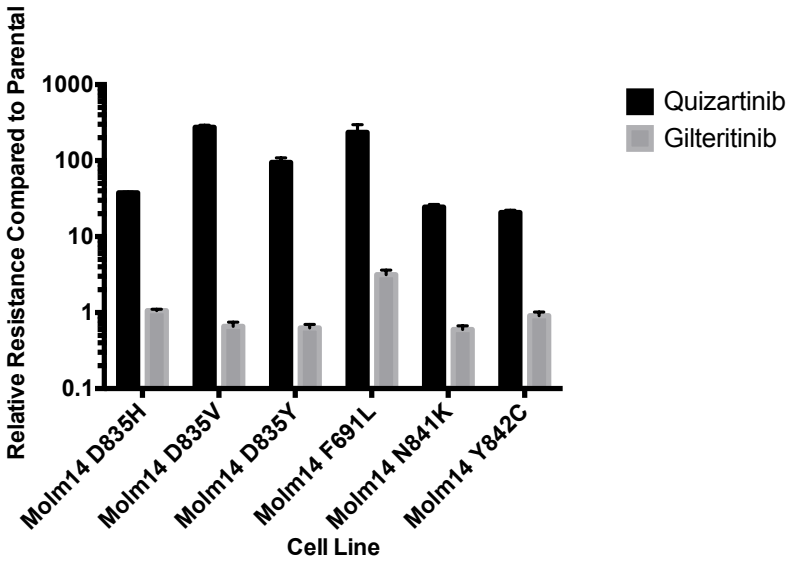

Figure S1. Gilteritinib Has Activity Against Quizartinib-Resistant Molm14 cell lines . Normalized cell viability of Molm14 cells containing indicated FLT3 mutations the after 48 hours in various concentrations of (A) quizartinib (B) gilterinib (error bars represent s.d. of triplicates from the same experiment). (C) Calculated inhibitory concentration 50 (IC50) of quizartinib and gilteritinib in Molm14 FLT3 mutant cell lines. (D) Relative resistance of indicated Molm14 mutant cell lines compared to parental Molm14 (IC50 of mutant/IC50 of parental) in the presence quizartinib and gilteritinib. (error bars represent s.d. of triplicate experiments).

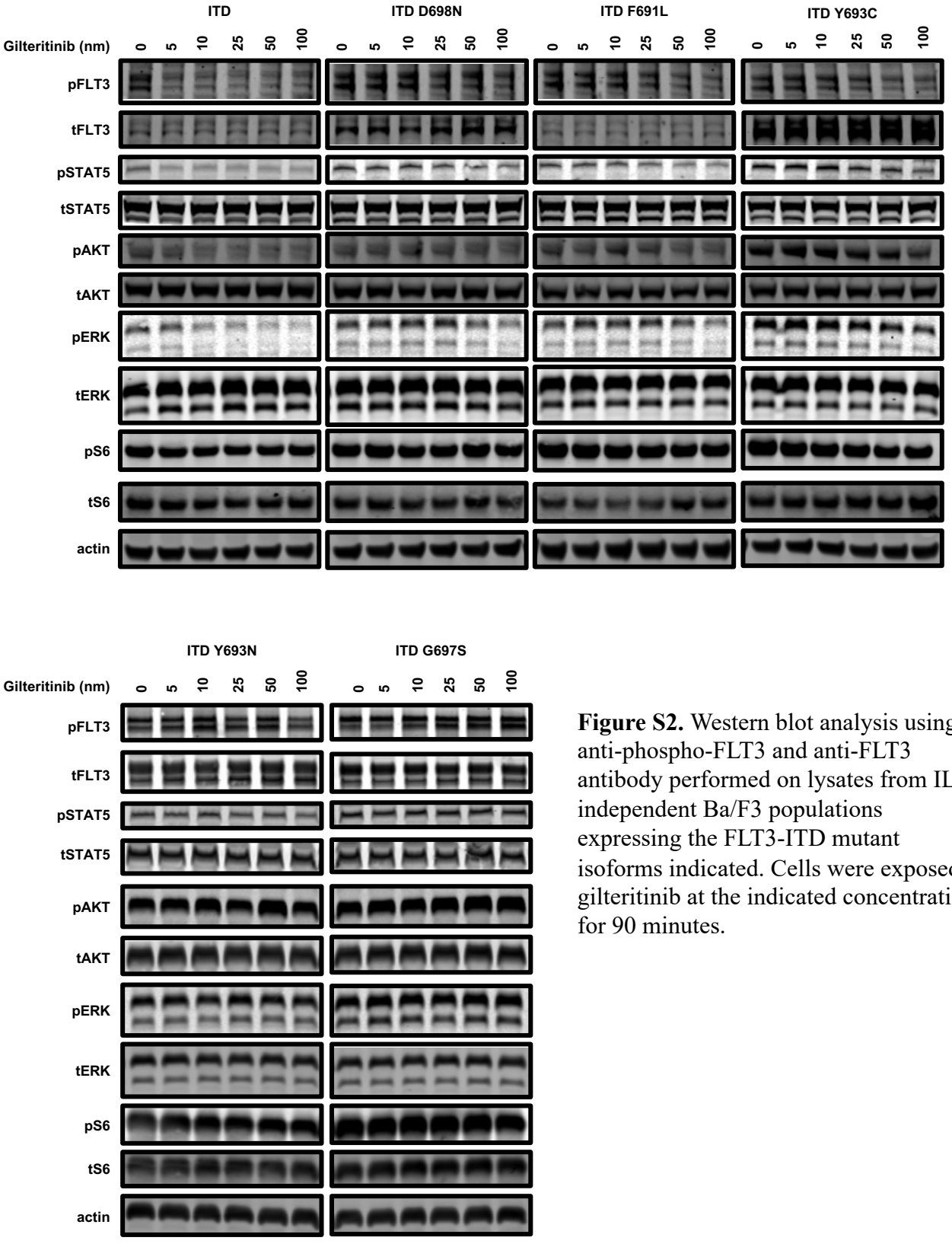

Figure S2. Western blot analysis using anti-phospho-FLT3 and anti-FLT3 antibody performed on lysates from IL-3-independent Ba/F3 populations expressing the FLT3-ITD mutant isoforms indicated. Cells were exposed to gilteritinib at the indicated concentrations for 90 minutes.

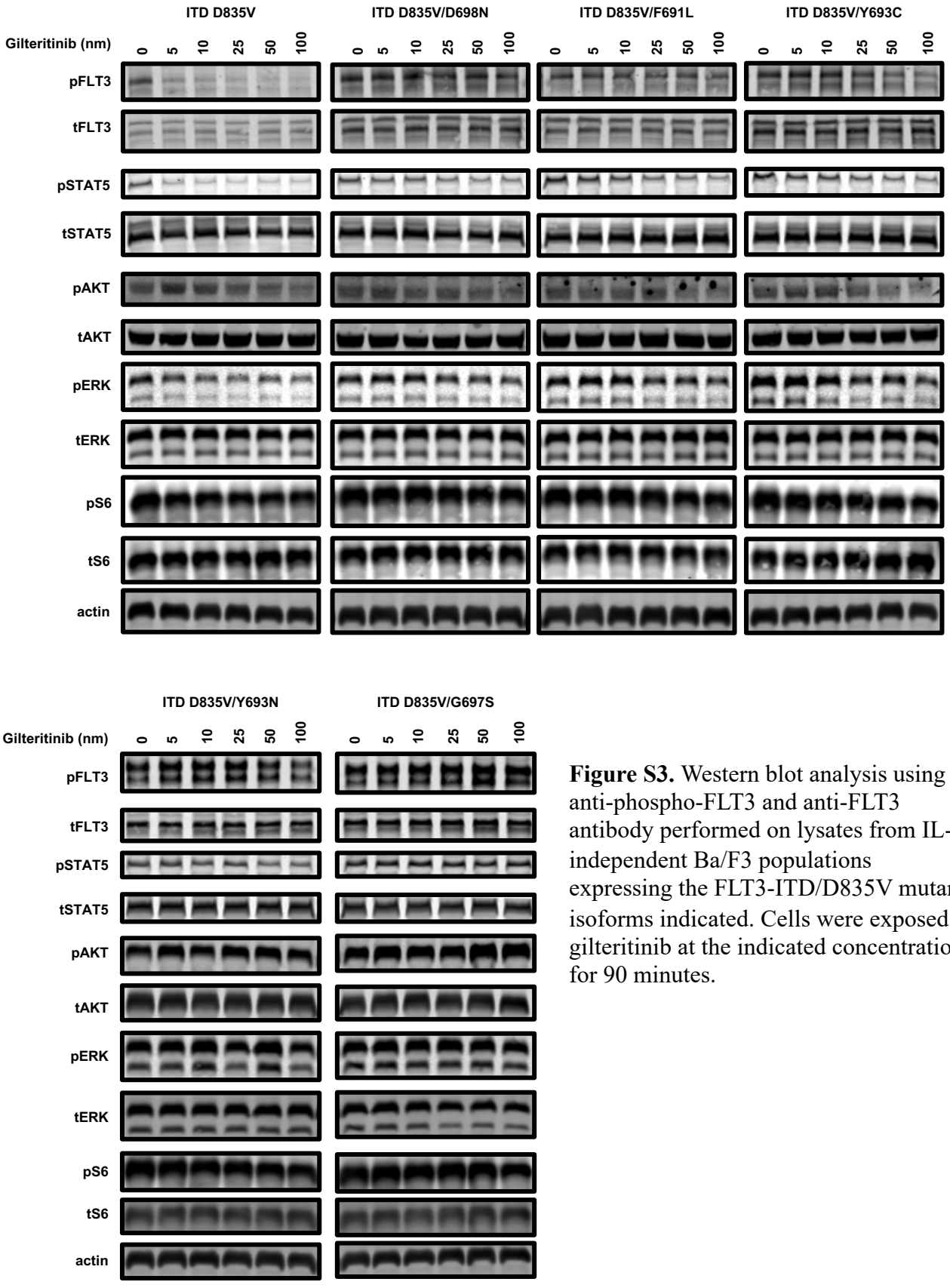

Figure S3. Western blot analysis using anti-phospho-FLT3 and anti-FLT3 antibody performed on lysates from IL-3-independent Ba/F3 populations expressing the FLT3-ITD/D835V mutant isoforms indicated. Cells were exposed to gilteritinib at the indicated concentrations for 90 minutes.