Supplementary material to:

Preclinical activity and a pilot phase I study of pacritinib, an oral JAK2/FLT3 inhibitor, and chemotherapy in *FLT3*-ITD-positive AML

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Methods

Exclusion criteria

Patients receiving any other investigational agents or that have received other investigational agents within 14 days of enrollment; patients with significantly decreased or obstructed gastrointestinal tract; patients with serious medical psychiatric illness that could interfere with participation; patients who had chemotherapy or radiotherapy or major surgery within 2 weeks prior to entering the study; patients with active central nervous system malignancy; patients with a history of platelet alloimmunization; patients with other malignancy within the last 3 years, other than curatively treated basal cell or squamous cell skin cancer, carcinoma in situ of the cervix, organ confined or treated nonmetastatic prostate cancer with negative prostate-specific antigen, in situ breast carcinoma after complete surgical resection, or superficial transitional cell bladder carcinoma; patients who are not able to swallow capsules or tablets; known active HIV or hepatitis A, B, or C virus infection

Deep amplicon sequencing

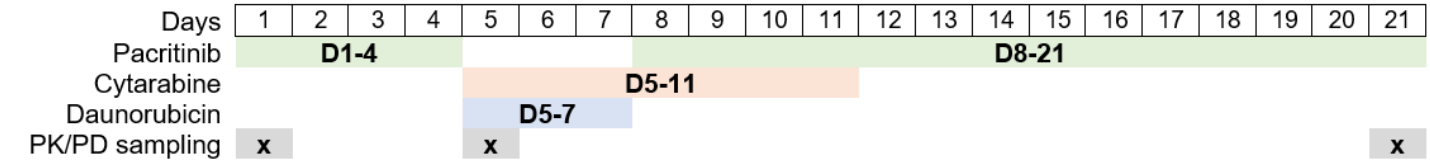
Briefly, FLT3 exon 17 was amplified from gDNA using forward primer 5'-TGAACGCAACAGCTTATGGA3' and reverse primer 5'-CCATGAAGCCCTGAGATT TG -3'. FLT3 exon 20 was amplified using forward primer 5'- TTCCATCACCGGTACCTCCTA -3' and reverse primer 5'-

CCTGAAGCTGCAGAAAAACC -3'. PCR amplicons were cleaned up using QIAquick PCR Purification Kit (Qiagen) and DNA concentrations were determined using an Invitrogen Quibit 3 Fluorometer and Qubit dsDNA BR Assay Kit. 0.1 ng input DNA from each exon was fragmented, tagged with adapters, and libraries were prepared using the Nextera XT DNA Sample Preparation Kit following the manufacturer's instructions (Illumina). Libraries were normalized and pooled using manufacturer's protocol then sequenced on an Illumina MiSeq System (Illumina) with 150bp paired-end reads. Image analyses and base calling were conducted using MiSeq Control Software versions 1.5.15.1 or 2.2 and Real Time Analysis versions 1.13.148 or 1.17.28. After removing the adapter sequences, high-quality reads (Phred-like score Q30 or greater) with at least 50 nucleotides were aligned to human *FLT3* reference sequence (UCSC hg19). Using CLC Genomics Workbench v11 (CLCBio) Mutations were detected and the frequencies of mutation were determined using Integrative genomics (IVG; Broad Institute). The minimum frequency of mutation detection at each genomic location was set with a threshold at the upper limit of the 99.9% confidence interval from the maximum sequencing error rate representing the platform sensitivities for detecting the low frequency allele with corresponding substitution.

Targeted Gene Panel

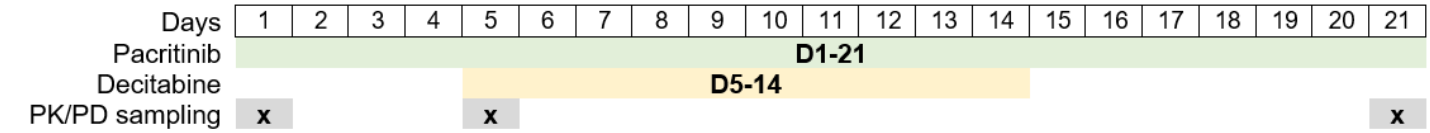
Genomic DNA isolated from blood leukocytes or bone marrow was profiled using digital droplet PCR method (Thunderbolt panel, Raindance Technologies) using the MiSeq Illumina platform. Analysis of the neoplasm-associated variants in the 49 genes was included in the targeted gene panel. UCSC hg19, NextGENe software and the GenomOncology platform were utilized for analysis along with a pathologist interpretation.

Cohort A



Dose level	Pacritinib (mg PO)	Cytarabine (mg/m^2 q24h)	Daunorubicin (mg/m^2 q24h)	N
-2	200 (100 BID)	100	60	5
-1	300 (200 AM, 100PM)	100	60	
1	400 (200 BID)	100	60	

Cohort B



Dose level	Pacritinib (mg PO)	Decitabine (mg/m^2 q24h)	N
-2	200 (100 BID)	20	6
-1	300 (200 AM, 100PM)	20	
1	400 (200 BID)	20	2

Supplementary Figure 1. Study schema and patient enrollment. Patients were enrolled in one of two cohorts. Each drug was administered on the indicated days (D).

Supplementary Table 1. Binding affinities of pacritinib and midostaurin to FLT3 and mutants.

	Kd (nM)	
FLT3	Pacritinib	Midostaurin
Wildtype	1.9	2.6
ITD	8.2	3.2
D835H	14	1.3
D835V	0.87	1.5
D835Y	8.5	1.4
ITD/D835V	0.69	2.3
ITD/F691L	17	2.7

Supplementary Table 2. Activity of pacritinib against FLT3 mutants in a kinase assay.

FLT3	IC50 (nM)
ITD	9.0
D835Y	3.1

Supplementary Table 3. Activity of pacritinib in Ba/F3 cells expressing FLT3 mutants.

FLT3	IC50 (nM)
GFP + IL3	824
ITD	133
D835H	97
D835Y	300
ITD/D835H	306
ITD/D835Y	434
ITDF691L	291

Supplementary Table 4. Activity of pacritinib and midostaurin in FLT3-ITD+ AML cell lines.

	IC50 (nM)
Cell line	Pacritinib
MOLM13	73
MOLM13-RES (ITD/D835Y)	173
MV4-11	33

Supplementary Table 5. Ex vivo activity of pacritinib in primary FLT3-ITD+ AML blast samples.

	IC50 (nM)	
	Pacritinib	Midostaurin
FLT3-ITD/IDH2-R140Q murine blasts	8700	10700
Patient 5	290	470
Patient 6	152	460
Patient 9	302	250

Supplementary Table 6. Complete list of toxicity in all cohorts

	Cohort A: Pacaritinib 200mg, cytarabine, 100mgm2, Daunorubicin 60mg/m2 (n = 5)			Cohort B dose 1: Pacritinib 200mg, Decitabine 20mg/m2 (n = 6)			Cohort B Dose 2: Pacritinib 400mg, Decitabine 20mg/m2 (n = 2)			Overall (n = 13)		
	1&2	3+	Total (n = 5)	1&2	3+	Total (n = 6)	1&2	3+	Total (n = 2)	1&2	3+	Total (n = 13)
Blood and lymphatic system disorders												
ANEMIA	0	3 (60)	3 (60)	1 (16.7)	5 (83.3)	6 (100)	1 (50)	1 (50)	2 (100)	2 (15.4)	9 (69.2)	11 (84.6)
ACTIVATED PARTIAL THROMBOPLASTIN TIME PROLONGED	1 (20)	0	1 (20)	1 (16.7)	0	1 (16.7)	0	0	0	2 (15.4)	0	2 (15.4)
AUTOIMMUNE HEMOLYTIC REFRACTORY ANEMIA	0	1 (20)	1 (20)	0	0	0	0	0	0	0	1 (7.7)	1 (7.7)
FEBRILE NEUTROPENIA	0	3 (60)	3 (60)	0	1 (16.7)	1 (16.7)	0	0	0	0	4 (30.8)	4 (30.8)
INR INCREASED	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
LYMPHOCYTE COUNT DECREASED	1 (20)	2 (40)	3 (60)	1 (16.7)	2 (33.3)	3 (50)	0	1 (50)	1 (50)	2 (15.4)	5 (38.5)	7 (53.8)
LYMPHOCYTE COUNT INCREASED	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
NEUTROPHIL COUNT DECREASED	0	2 (40)	2 (40)	0	3 (50)	3 (50)	0	1 (50)	1 (50)	0	6 (46.2)	6 (46.2)
PLATELET COUNT DECREASED	0	5 (100)	5 (100)	0	5 (83.3)	5 (83.3)	0	1 (50)	1 (50)	0	11 (84.6)	11 (84.6)
WHITE BLOOD CELL DECREASED	0	4 (80)	4 (80)	0	3 (50)	3 (50)	0	1 (50)	1 (50)	0	8 (61.5)	8 (61.5)
Cardiac disorders												
ASYSTOLE	0	0	0	0	1 (16.7)	1 (16.7)	0	0	0	0	1 (7.7)	1 (7.7)
ELECTROCARDIOGRAM QT CORRECTED INTERVAL PROLONGED	4 (80)	0	4 (80)	1 (16.7)	1 (16.7)	2 (33.3)	0	0	0	5 (38.5)	1 (7.7)	6 (46.2)
PERICARDIAL EFFUSION	0	0	0	0	1 (16.7)	1 (16.7)	0	0	0	0	1 (7.7)	1 (7.7)
SINUS TACHYCARDIA	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (7.7)	0	1 (7.7)
Gastrointestinal disorders												
ALANINE AMINOTRANSFERASE INCREASED	0	0	0	0	1 (16.7)	1 (16.7)	0	0	0	0	1 (7.7)	1 (7.7)
BLOOD BILIRUBIN INCREASED	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (7.7)	0	1 (7.7)
CONSTIPATION	0	0	0	1 (16.7)	0	1 (16.7)	1 (50)	0	1 (50)	2 (15.4)	0	2 (15.4)
DIARRHEA	3 (60)	0	3 (60)	1 (16.7)	0	1 (16.7)	0	0	0	4 (30.8)	0	4 (30.8)
GUM BLEEDING	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (7.7)	0	1 (7.7)
MUCOSITIS ORAL	2 (40)	0	2 (40)	1 (16.7)	1 (16.7)	2 (33.3)	0	0	0	3 (23.1)	1 (7.7)	4 (30.8)
NAUSEA	3 (60)	0	3 (60)	3 (50)	0	3 (50)	0	1 (50)	1 (50)	6 (46.2)	1 (7.7)	7 (53.8)
ORAL HEMORRHAGE	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
RECTAL PAIN	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
VOMITING	0	0	0	2 (33.3)	0	2 (33.3)	1 (50)	0	1 (50)	3 (23.1)	0	3 (23.1)
General disorders and administration site conditions												
CHILLS	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
EDEMA LIMBS	1 (20)	0	1 (20)	1 (16.7)	0	1 (16.7)	0	0	0	2 (15.4)	0	2 (15.4)
FATIGUE	3 (60)	0	3 (60)	2 (33.3)	0	2 (33.3)	0	0	0	5 (38.5)	0	5 (38.5)
NIGHT SWEATS	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
Infections and infestations												
CATHETER RELATED INFECTION	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
GRAM NEGATIVE BACTERIA	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
LUNG INFECTION	0	1 (20)	1 (20)	0	1 (16.7)	1 (16.7)	0	0	0	0	2 (15.4)	2 (15.4)
PAPULOPUSTULAR RASH	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
SEPSIS	0	1 (20)	1 (20)	0	0	0	0	0	0	0	1 (7.7)	1 (7.7)
UPPER RESPIRATORY INFECTION	0	0	0	0	1 (16.7)	1 (16.7)	0	0	0	0	1 (7.7)	1 (7.7)

URINARY TRACT INFECTION	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
Injury, poisoning and procedural complications												
FALL	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (7.7)	0	1 (7.7)
Metabolism and nutrition disorders												
ANOREXIA	0	0	0	2 (33.3)	0	2 (33.3)	0	0	0	2 (15.4)	0	2 (15.4)
HYPERGLYCEMIA	1 (20)	0	1 (20)	1 (16.7)	0	1 (16.7)	1 (50)	0	1 (50)	3 (23.1)	0	3 (23.1)
HYPERMAGNESEMIA	1 (20)	0	1 (20)	0	0	0		0	0	1 (7.7)	0	1 (7.7)
HYPERURICEMIA	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (7.7)	0	1 (7.7)
HYPOALBUMINEMIA	2 (40)	0	2 (40)	1 (16.7)	0	1 (16.7)	0	0	0	3 (23.1)	0	3 (23.1)
HYPOCALCEMIA	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
HYPOKALEMIA	1 (20)	0	1 (20)	2 (33.3)	0	2 (33.3)	0	1 (50)	1 (50)	3 (23.1)	1 (7.7)	4 (30.8)
HYPONATREMIA	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
HYPOPHOSPHATEMIA	0	1 (20)	1 (20)	0	1 (16.7)	1 (16.7)	0	0	0	0	2 (15.4)	2 (15.4)
Musculoskeletal and connective tissue disorders												
SHOULDER PAIN (LEFT)	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
PAIN IN EXTREMITY	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
Nervous system disorders												
AKATHISIA	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (7.7)	0	1 (7.7)
DYSGEUSIA	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
FACIAL NERVE DISORDER	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
HEADACHE	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
PARESTHESIA	1 (20)	0	1 (20)				0	0	0	1 (7.7)	0	1 (7.7)
PERIPHERAL MOTOR NEUROPATHY	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
TREMOR	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
Psychiatric disorders												
INSOMNIA	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (7.7)	0	1 (7.7)
Renal and urinary disorders												
URINARY FREQUENCY	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
URINARY TRACT OBSTRUCTION	0	0	0	0	1 (16.7)	1 (16.7)	0	0	0	0	1 (7.7)	1 (7.7)
URINARY URGENCY	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
Respiratory, thoracic and mediastinal disorders												
COUGH	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
DYSPNEA	1 (20)	0	1 (20)	0	0	0	1 (50)	0	1 (50)	2 (15.4)	0	2 (15.4)
EPISTAXIS	0	0	0	0	0	0	1 (50)	0	1 (50)	1 (7.7)	0	1 (7.7)
HYPOXIA	0	2 (40)	2 (40)	0	0	0	0	0	0	0	2 (15.4)	2 (15.4)
PLEURAL EFFUSION	1 (20)	0	1 (20)	1 (16.7)	0	1 (16.7)	0	0	0	2 (15.4)	0	2 (15.4)
RESPIRATORY FAILURE	0	2 (40)	2 (40)	0	1 (16.7)	1 (16.7)	0	0	0	0	3 (23.1)	3 (23.1)
SORE THROAT	0	0	0	1 (16.7)	0	1 (16.7)	0	0	0	1 (7.7)	0	1 (7.7)
Skin and subcutaneous tissue disorders												
BULLOUS DERMATITIS	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
PRURITUS	4 (80)	0	4 (80)	0	0	0	0	0	0	4 (30.8)	0	4 (30.8)
RASH ACNEIFORM	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)
RASH MACULO-PAPULAR	1 (20)	1 (20)	2 (40)	0	0	0	0	0	0	1 (7.7)	1 (7.7)	2 (15.4)
RASH	2 (40)	0	2 (40)	1 (16.7)	0	1 (16.7)	0	0	0	3 (23.1)	0	3 (23.1)
Vascular disorders												
HYPERTENSION	1 (20)	1 (20)	2 (40)	0	0	0	1 (50)	0	1 (50)	2 (15.4)	1 (7.7)	3 (23.1)
HYPOTENSION	1 (20)	0	1 (20)	0	0	0	0	0	0	1 (7.7)	0	1 (7.7)

Supplementary Table 7. List of mutations in *FLT3* exons 17 and 20 by deep amplicon sequencing.

		FLT3 Exon 17					*FLT3* Exon 20								
Pt	Time point	F691 WT (TTT)	F691L (TTT) C (F>L)	F691L (TTT) G/A (F>L)	F691L (TTT) C (F>L) (%)	F691L (TTT) G/A (F>L) (%)	D835 WT (GAT)	D835Y (GAT) T D>Y	D835H (GAT) C D>H	D835N (GAT) A D>N	D835V (GAT) T D>V	D835Y (%)	D835H (%)	D835N (%)	D835V (%)
1	C1D5	469569	51	73	0.011	0.015	806999	148	59	129	159	0.02	0.01	0.02	0.02
2	Screen	485142	51	61	0.011	0.012	730394	135	41	129	136	0.02	0.01	0.02	0.02
3	Screen	563490	94	112	0.017	0.020	770481	219	76	166	220	0.03	0.01	0.02	0.03
4	C1D5	560214	51	68	0.009	0.012	798014	150	44	96	120	0.02	0.01	0.01	0.02
5	Screen	493229	44	76	0.009	0.015	801057	283	41	101	136	0.04	0.01	0.01	0.02
6	Screen	578245	69	97	0.012	0.017	782173	169	51	122	152	0.02	0.01	0.02	0.02
7	Screen	557796	56	95	0.010	0.017	786839	175	59	118	155	0.02	0.01	0.01	0.02
8	Screen	550743	69	86	0.013	0.015	722458	173	67	421	154	0.02	0.01	0.06	0.02
9	Screen	511748	89	167	0.017	0.032	777473	17952	104	208	247	**2.3**	0.01	0.03	0.03
10	Screen	524412	42	70	0.008	0.013	739752	148	44	100	139	0.02	0.01	0.01	0.02
11	Screen	510672	95	116	0.019	0.023	738506	272	108	166	237	0.04	0.01	0.02	0.03
12	Screen	555086	74	115	0.013	0.021	759971	293	112	230	237	0.04	0.01	0.03	0.03
13	Screen	588343	88	154	0.015	0.026	717111	276	102	234	287	0.04	0.01	0.03	0.04

Data are number of reads at each position, unless indicated otherwise.

Supplementary Table 8. List of other mutations from a targeted gene panel and percentage blast change from baseline to day 5 of course 1 (C1D5).

		% Blast (PB)		% Blast (BM)	
Pt	Other mutations (VAF)	Baseline	C1D5	Baseline	C1D5
1		0	9	2	NA
2	RUNX1 (0.25), TET2 (0.29)	17	NA	35	NA
3	NPM1 (0.23), WT1 (0.26), DNMT3A (0.26)	32.2	6.8	45	NA
4	WT1 (0.32), TET2 (0.48)	0	10.4	NA	NA
5	NPM1 (0.43), IDH2 (0.44)	39	30	31	NA
6	ASXL1 (0.46)	52	71	80	NA
7	NPM1 (0.40), TET2 (T965fs, 0.416; Y1245fs, 0.45)	69	65	NA	NA
8	IDH2 (0.50)	35.9	29.3	65	NA
9	NPM1 (0.41)	49.7	15	58	NA
10		88	20	88	NA
11	NPM1 (0.43), DNMT3A (0.46)	10	0	NA	NA
12		0	NA	NA	NA
13	CBL (0.34)	14	NA	19.7	NA

Abbreviations: VAF, variant allele fraction; PB, peripheral blood; BM, bone marrow.