Luxeptinib (CG-806) Targets FLT3 and Clusters of Kinases Operative in Acute Myeloid Leukemia

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ABSTRACT

Luxeptinib (CG-806) simultaneously targets FLT3 and select other kinase pathways operative in myeloid malignancies. We investigated the range of kinases it inhibits, its cytotoxicity landscape *ex vivo* with acute myeloid leukemia (AML) patient samples, and its efficacy in xenograft models. Luxeptinib inhibits wild-type (WT) and many of the clinically relevant mutant forms of FLT3 at low nanomolar concentrations. It is a more potent inhibitor of the activity of FLT3—internal tandem duplication, FLT3 kinase domain and gatekeeper mutants than against WT FLT3. Broad kinase screens disclosed that it also inhibits other kinases that can drive oncogenic signaling and rescue pathways, but spares kinases known to be associated with clinical toxicity. *In vitro* profiling of luxeptinib against 186 AML fresh patient samples demonstrated greater potency relative to other FLT3 inhibitors, including cases with mutations in *FLT3*, isocitrate dehydrogenase-1/2, *ASXL1, NPM1, SRSF2, TP53,* or *RAS*, and activity was documented in a xenograft AML model. Luxeptinib administered continuously orally every 12 hours at a dose that yielded a mean C_{min} plasma concentration of 1.0 ± 0.3 μmol/L (SEM) demonstrated strong antitumor activity but no myelosuppression or evidence of tissue damage in mice or dogs in acute toxicology studies. On the basis of these studies, luxeptinib was advanced into a phase I trial for patients with AML and myelodysplastic/myeloproliferative neoplasms.

Introduction

Myeloid malignancies demonstrate extraordinary plasticity and survive by dysregulation of receptors, transcription factors, tumor suppressors, pro-survival pathways, epigenetic processes, altered metabolic activities, and other aberrations. The most commonly mutated gene in acute myeloid leukemia (AML) is the *FLT3* receptor tyrosine kinase, which can harbor an internal tandem duplication (ITD) and/or point mutations in the kinase domain that constitutively activate its activity. These mutations in *FLT3* occur in approximately 30% of AML patients and are associated with increased risk of relapse and poor survival (1–3). Drugs with high selectivity for FLT3 now play central roles in the treatment of myeloid malignancies. However, their benefit is regularly subverted by the occurrence of additional mutations in FLT3 or through activation of rescue pathways that compensate for its survival function.

The FLT3 inhibitor gilteritinib provides an example of this phenomenon. Gilteritinib produced a response rate of 34% in FLT3 inhibitor-naïve patients with relapsed or refractory *FLT3*-mutated AML patients but the event-free survival was only 2.8 months and recurrence was associated with mutations in *NRAS, KRAS*, and others that activate the MAPK signaling pathway (4, 5). Similar results of clonal evolution towards tumors with mutation of *RAS* and/or additional pathways were reported as the primary mechanism of relapse to another FLT3 inhibitor, crenolanib (6). While *FLT3* mutations dominate in AML driven by the mutant forms of this enzyme, other receptor tyrosine kinases, their ligands and a host of intracellular signaling pathways, including RAS/MAPK and AURK, mediate resistance to inhibitors of FLT3 or other important targets for AML, such as BCL2 (5–10). Collectively, these circumstances highlight the need for a more panoptic approach to the dilemma of resistance in *FLT3*-driven myeloid malignancies.

Luxeptinib is an oral non-covalent kinase inhibitor that very potently inhibits the wild-type (WT) and mutant forms of FLT3 but also selectively suppresses specific clusters of tyrosine and serine-threonine kinases that can participate in rescue pathways. Luxeptinib is the first molecule advanced into clinical trials that potently inhibits both FLT3 and many of the kinases that participate in rescue pathways. The hypothesis underlying its development is that simultaneous inhibition of both targets that are essential to survival and kinases in pathways whose activation can replace the function of the inhibited essential kinase will kill malignant cells resistant to many other agents and may reduce the rate at which further resistance emerges. We report here on the non-clinical studies that supported the advancement of luxeptinib into a phase Ia/b trial in patients with AML and myelodysplastic/myeloproliferative neoplasms (MDS/MPN) who are resistant, refractory, or intolerant to established therapies.

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Materials and Methods

Drugs

Luxeptinib was provided by Aptose Biosciences Inc. FLT3 inhibitors gilteritinib, quizartinib, crenolanib, and midostaurin were purchased from Chemietek, Selleck Chemicals, Cayman Chemical, and Sigma-Aldrich, respectively.

Kinase screens

Screens using the ATP K_m for each recombinant kinase were performed by Reaction Biology Corporation and a screen using 1 mmol/L ATP was performed by Carna Biosciences using the mobility shift assay, IMAP. Binding affinity studies were performed using the DiscoverX KINOMEscan.

Cell lines and antiproliferative assays

The human AML cell lines were obtained from ATCC, except that EOL-1, MOLM-13, MOLM-14, MUTZ-8, NOMO-1, and SKM-1 were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and cell cultures (RRID:CVCL_0098). The mouse Ba/F3 cell lines expressing *FLT3* mutants ITD, D835Y, ITD plus D835Y, ITD plus F691 L were a generous gift from Dr. Michael Andreeff at the MD Anderson Cancer Center, Houston, TX. All lines were short tandem repeat—authenticated at ATCC or Leibniz Institute DSMZ-German Collection of Microorganisms and cell cultures. *Mycoplasma* testing was conducted every 2 months using the Universal Mycoplasma Detection Kit from ATCC (Catalog no. 30–1012K). The last *Mycoplasma* test was within 2 months of the last experiment with any given cell line. The cells were cultured in the medium recommended by the source for each cell line. Antiproliferative assays were performed using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, catalog no. G3581). Cells were exposed in four replicates to increasing concentrations of drugs for 72 hours. Studies using Ba/F3 cells expressing WT and mutant forms of BTK were performed by Advanced Cellular Dynamics, Inc, Seattle, WA.

Western blot analysis of kinase inhibition

Cells were treated with vehicle DMSO or luxeptinib for 1 or 24 hours and then lysed. Western blot analyses were performed with antibodies capable of detecting total and site-specifically phosphorylated forms of each kinase purchased from Cell Signaling Technology. Catalog numbers were as follows: FLT3 pY589/591 (RRID:AB_2049358); STAT5, 94205; STAT5 pY694; ERK1/2, 9107; ERK1/2 pT202/204, 9101, and PARP, 9532. Antibody to FLT3 was purchase from Abcam (#AB89554).

Xenograft

A dose-ranging xenograft study was performed in BALB/c nu/nu mice by Covance Inc as per company protocols. Luxeptinib was co-micronized with 2.5% w/w sodium lauryl sulfate for oral gavage; this is the same formulation as used in the phase I trial of luxeptinib. MV4–11 cells were inoculated subcutaneous in Matrigel (5×10^6 cells/mouse). Treatment was started when tumors reached 178 mm^3. Luxeptinib was administered twice a day by gavage for 28 days. In the MV4–11 xenograft study, treatment was restarted at a dose of 300 mg/kg twice a day if a tumor regrew after the first 28 days of treatment. Tumor size was measured by caliper every day and volume was calculated from bi-dimensional diameters using the formula $V = 0.5(L \times W^2)$, where L = larger of measured diameter and W = smaller of perpendicular diameter. Mice were euthanized if tumor volume reached >2,000 mm^3.

Primary AML sample testing

Drug sensitivity testing, DNA sequencing and RNA sequencing (RNA-seq) analysis of primary patient samples was carried out by investigators at Oregon Health & Science University. All patients gave written informed consent to participate in this study, which had the approval and guidance of the institutional review boards at Oregon Health & Science University (OHSU), University of Utah, University of Texas Medical Center (UT Southwestern), University of Miami, University of Colorado, University of Florida, NIH, and University of Kansas (KUMC). Samples were sent to the coordinating center (OHSU; IRB#9570; #4422; NCT01728402) where they were coded and processed. Detailed information on the procedures used has been published (3) and are presented in summary form here.

Ex vivo drug screen

Primary mononuclear cells were freshly isolated from peripheral blood and bone marrow of patients with AML and plated at 10,000 cells per well in 384-well plates. Drug stocks were prepared in DMSO and a seven-point dilution series (3-fold dilution steps, 10 μmol/L 0.0137 μmol/L) added to the primary patient samples. Under the culture conditions, vehicle-treated cells retain viability (>90%), but do not proliferate. After 3 days incubation, cell growth was measured by MTS assay, raw absorbance values were background subtracted then compared with untreated control wells to assess cell viability.

Whole-exome sequencing

Cell pellets from freshly isolated mononuclear cells were used to prepare genomic DNA using Qiagen kits. Whole-exome sequencing (WES) was performed using Illumina Nextera Rapid Capture Exome capture probes as per manufacturer's instructions.

RNA-seq

Cell pellets from freshly isolated mononuclear cells were used to prepare RNA using Qiagen kits. Libraries were prepared from poly(A) selected RNA using Agilent SureSelect Strand-Specific RNA library (RRID:SCR_019473) and sequenced on the Illumina HiSeq 2500 using 100 base pair paired-end reads.

Cytotoxic interaction between luxeptinib and venetoclax

Primary patient cells were cultured for 72 hours with either increasing concentrations of luxeptinib or venetoclax individually or at a fixed molar ratio (11–13).

Data availability statement

All raw and processed sequencing data, along with relevant clinical annotations can be found in dbGaP and Genomic Data Commons and are publicly available. The dbGaP accession ID is phs001657.v1.p1. Additional data can be found at www.vizome.org and in Tyner and colleagues (3).

Results

Luxeptinib potently inhibits WT and mutant FLT3

The structure of luxeptinib is shown in **Fig. 1A**. It is a non-covalent (reversible), ATP-competitive inhibitor of both WT and mutant forms of FLT3 at low or sub-nanomolar concentrations. Luxeptinib was screened against a broad range of kinases with enzyme inhibition assays at the ATP K_m for each kinase or at 1 mmol/L ATP, as well as with competition binding affinity studies. These studies revealed that

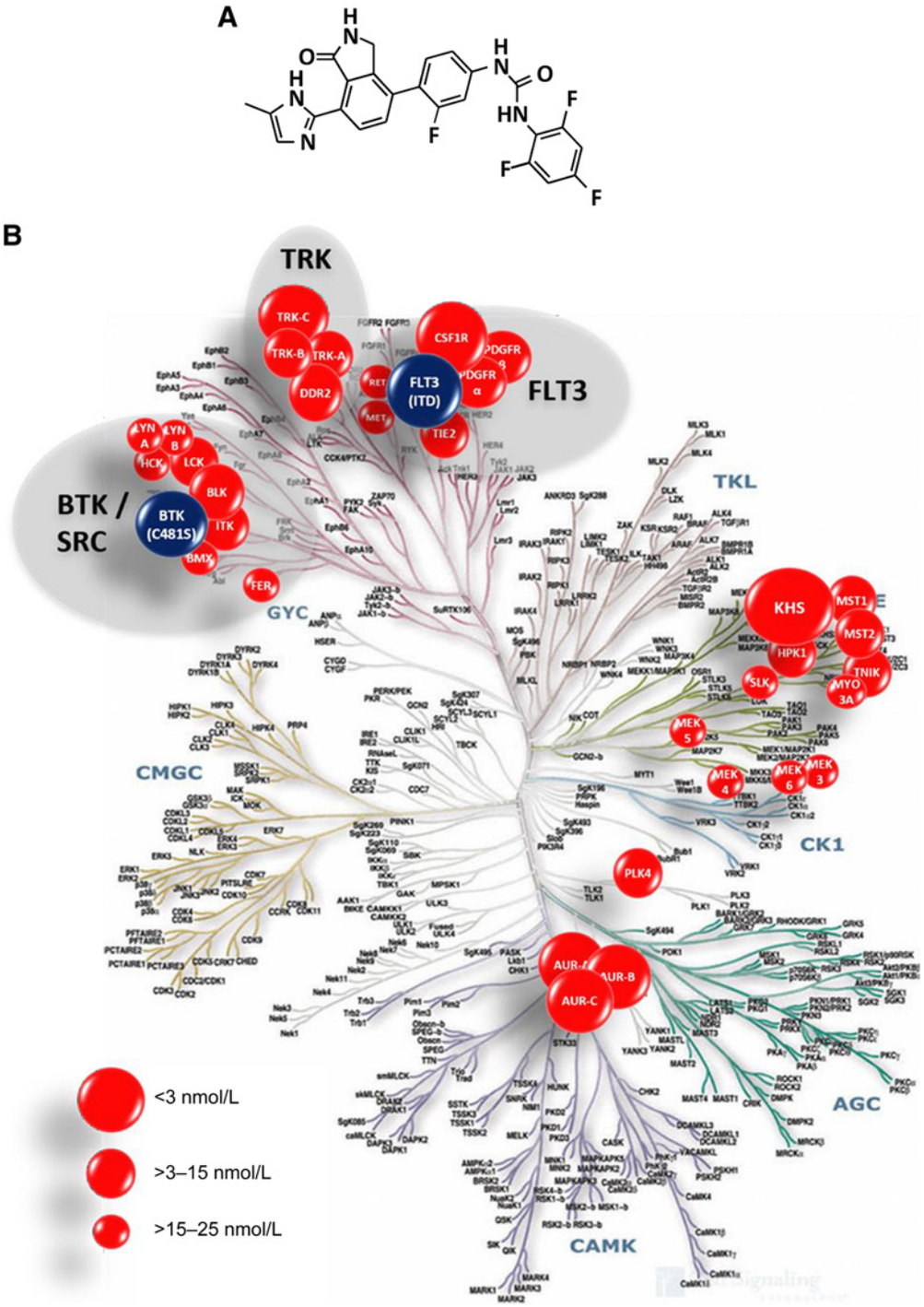


Figure 1.
Structure of luxeptinib and pattern of its kinase inhibition. **A,** Structure of luxeptinib. **B,** Principle kinases inhibited by luxeptinib as determined from broad kinase screens.

luxeptinib inhibits clusters of related kinases at low nanomolar concentrations (**Fig. 1B**; Supplementary Tables S1 and S2). Targeted receptor tyrosine kinases include the FLT3-containing type III receptor tyrosine kinase cluster (all WT and mutant forms of FLT3 plus CSF1R, TIE2, PDGFRα and PDGFRβ), the NTRK cluster (NTRK-1/2/3, DDR2, RET, and MET), as well as certain clusters of intracellular tyrosine kinases, including the family of kinases related to the TEC kinase (all forms of, BMX, and ITK), the closely associated SRC

Table 1. Potency of luxeptinib against key kinases.

Kinase	IC_{50} (nmol/L)[a]
FLT3-WT	8.7
FLT3-ITD	0.8
TEC	139
EGFR	>1,000
ERBB	>1,000

[a]Determined using recombinant kinase with ATP concentration at the K_m.

family kinases (LYN, YES, BLK, HCK, LCK, and FER), and certain serine/threonine kinases in the AURK-A/B/C cluster and the STE/MAPK cluster of the human kinome tree.

Testing against recombinant forms of FLT3 and other clinically important kinases demonstrated that luxeptinib inhibits WT and ITD mutant FLT3 (IC_{50} = 8.7 nmol/L and 0.8 nmol/L, respectively; **Tables 1** and **2**) as well as clinically important kinase domain mutations of FLT3 in residues D835, D841 (14), and the gatekeeper residue, F691, which can engender resistance to other FLT3 inhibitors (5, 6). Importantly, kinases targeted by luxeptinib do not include the TEC kinase itself, despite inhibiting other TEC family kinases, EGFR, or ERBB2, the inhibition of which can be associated with cardiac, skin and bleeding adverse events (15–19). Efforts to generate crystal structures with luxeptinib and various forms of the FLT3 kinase were unsuccessful. Against a panel of 10 malignant myeloid AML cell lines, luxeptinib demonstrated antiproliferative IC_{50} values that ranged from sub-nanomolar to low micromolar concentrations, consistent with its potent inhibition of the FLT3 kinase and its mutants (**Table 3**), as well as its potency against EOL-1 AML cells, which are driven by the FIP1L1-PDGFRA fusion protein.

Luxeptinib also was highly effective relative to other FLT3 inhibitors (quizartinib, gilteritinib, and crenolanib), against a panel of murine Ba/F3 cells whose survival was dependent on clinically relevant forms of mutant FLT3 (ITD, D835Y, ITD + D835Y, ITD + F691L; **Fig. 2A**; **Table 4**). The fact that luxeptinib retains activity against FLT3 mutants that render cells resistant to quizartinib, gilteritinib, and crenolanib may be of potential clinical significance. **Figure 2B** documents that luxeptinib potently reduces the phosphorylation of FLT3 but also reduces the phosphorylation of downstream STAT5 and ERK1/2 indicating decreased activity of pathways that contribute to bypass pathway-mediated resistance to FLT3 inhibitors. Luxeptinib induced apoptosis in all 3 FLT3-mutant AML cell lines and the PDGFR-α mutant EOL-1 cell line tested at concentrations of 5 nmol/L or less, as documented by PARP cleavage (**Fig. 2C**).

Table 2. Binding affinity of luxeptinib to WT and mutant forms of FLT3.

FLT3 proteins (fragments)	K_d (nmol/L)
FLT3–WT	0.24
FLT3– ITD	3.1
FLT3 D835Y (TKD)	4.2
FLT3 D835H (TKD)	2.2
FLT3 D835V (TKD)	7.9
FLT3 D833Q (TKD)	6.4
FLT3 D841I (TKD)	0.8
FLT3 K663Q (TKD)	0.55
FLT3 ITD/F691L	16

Table 3. Antiproliferative potency of luxeptinib against malignant human myeloid lines.

Cell Line	*FLT3* status	Mean IC_{50} (nmol/L)
EOL-1	WT	0.045
MV4–11	*FLT3*-ITD	0.238
MOLM-13	*FLT3*-ITD	0.395
NOMO-1	WT	20.52
KG-1	WT	28.69
HL60	WT	279.60
MUTZ-8	WT	793.70
SKM-1	WT	816.10
THP-1	WT	2,972.0
HEL92.1.7	WT	4,687.0

Sensitivity of AML cells from patient samples to luxeptinib

Through a collaboration with the Beat AML 1.0 Initiative, luxeptinib was tested using an *ex vivo* cell viability assay to determine its IC_{50} against 364 freshly harvested samples from patients with AML and 31 from patients with MDS/MPN (3), and the median IC_{50} values for luxeptinib were <0.2 μmol/L for both AML and MDS/MPN patient samples (Supplementary Fig. S1). Against AML patient samples, luxeptinib was equally potent against cells from patients in the adverse, intermediate, and favorable risk groups (2017 ELN risk stratification), and against cells from patients with relapsed or transformed AML (World Health Organization classification), as for those from patients with de novo AML (**Fig. 3A** and **B**, respectively).

Luxeptinib and six other FLT3 inhibitors (including midostaurin, sorafenib, dovitinib, quizartinib, crenolanib, and gilteritinib) were also screened in the *ex vivo* cell viability assay against 186 primary samples from patients with AML representing a diverse array of AML subpopulations. An IC_{50} was derived for each drug and for each patient sample, and the IC_{50} data were transformed into a heat map. As shown in **Fig. 3C**, luxeptinib yielded IC_{50} values <0.1 μmol/L at a higher frequency than the other drugs (median IC_{50} for luxeptinib was 0.0945 μmol/L ($n = 186$, $P < 0.001$ relative to other FLT3 inhibitors). The mean IC_{50} values for luxeptinib differed from that of each of the other drugs for both FLT3-ITD negative and positive cases in two-sided paired comparisons with the exception of quizartinib in the FLT3-ITD positive cells (Supplementary Table S3).

Among agents emerging for the treatment of hematologic malignancies, venetoclax has proven to be impressive (20). The effectiveness of venetoclax alone, luxeptinib alone, and the combination of venetoclax and luxeptinib was determined for samples from patients with AML and MDS/MPN (**Fig. 3D**). Interestingly, venetoclax had a binary effectiveness profile, either having a very low IC_{50} or producing minimal to no cell killing; this characteristic was observed across both AML and MDS/MPN. In contrast, luxeptinib produced a more graded distribution of IC_{50} values. Importantly, a combination of the two agents in a fixed molar ratio enhanced cell killing of the majority of samples relative to either drug alone. These data support testing of the combination of venetoclax and luxeptinib in clinical trials.

Association of luxeptinib sensitivity with genetic features

Exploration of the association of luxeptinib sensitivity with gene abnormalities and expression levels in populations of WT and mutant samples was possible because WES ($n = 228$) and RNA-seq ($n = 111$) were performed on the malignant cells obtained from patients with AML from the Beat AML Initiative. Patient samples with FLT3-ITD

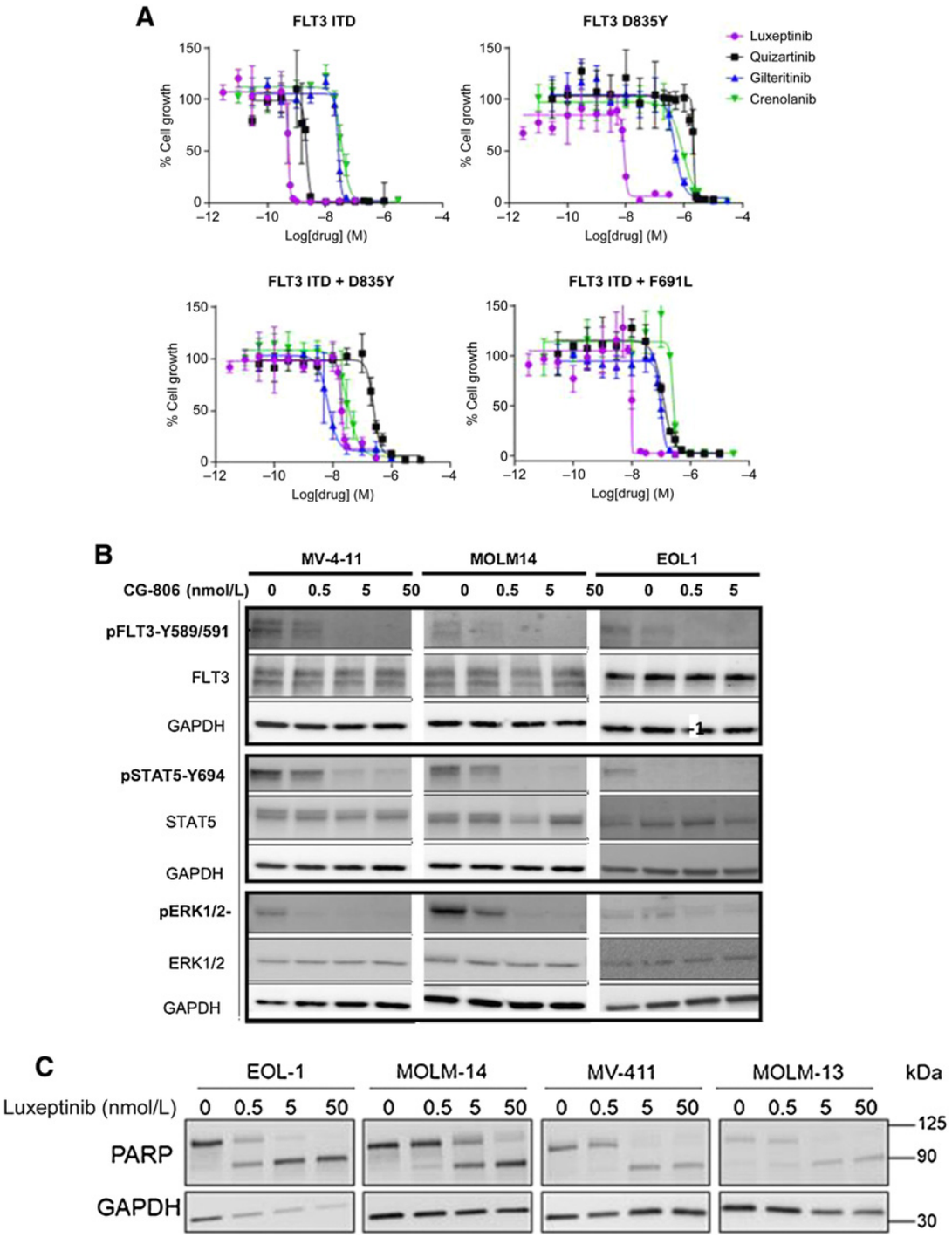


Figure 2.

Activity of luxeptinib against *FLT3*-mutant myeloid cell lines. **A,** Growth inhibition curves for luxeptinib, quizartinib, gilteritinib, and crenolanib against *FLT3*-mutant AML cell lines. **B,** Western blot analysis documenting concentration-dependent inhibition FLT3 and bypass pathways known to contribute to FLT3 inhibitor resistance (representative image from 3 biological repeats). **C,** Documentation of the ability of luxeptinib to kill FLT3-ITD cells as evidenced by induction of PARP cleavage.

mutations were more sensitive to luxeptinib (FDR-corrected $P < 0.01$) irrespective of the presence or absence of *NPM1* mutations (**Fig. 4A**), and there was a trend toward greater sensitivity in cases with high *FLT3-ITD* allelic ratio (**Fig. 4B**). Across all patients, there was no discernable loss of sensitivity caused by *ASXL1* or *TP53* mutations (**Figs. 4C** and **D**, respectively). Unexpectedly, specimens containing isocitrate dehydrogenase-1 (*IDH1*) R132 mutations were more sensitive to luxeptinib relative to those with WT *IDH1*, although this did

Table 4. IC_{50} in Ba/F3 cells transfected with FLT3 variants.

FLT3 Inhibitor	IC_{50}, nmol/L[a]			
	FLT3-ITD	FLT3 D835Y	FLT3-ITD + D835Y	FLT3-ITD + F691L
CG-806	1 ± 0.2	6 ± 0.4	22 ± 9	9 ± 1
Quizartinib	2 ± 0.4	2138 ± 261	269 ± 27	144 ± 20
Gilteritinib	28 ± 2	521 ± 68	9 ± 5	96 ±6
Crenolanib	38 ± 8	831 ± 195	37 ± 13	286 ± 9

[a]Mean ± SD, $n = 3$.

not reach statistical significance (**Fig 4E**); this was not the case for *IDH2*. Luxeptinib was significantly more potent than gilteritinib, quizartinib or midostaurin whether or not the sample contained an NRAS mutation and irrespective of what other mutations might or might not be present, when all four drugs were tested against the same samples (**Fig. 4F–G**). These results indicate that luxeptinib is highly potent against WT as well as mutant *FLT3*, and against AML cells containing a broad array of mutations commonly found in AML including in *NPM1, ASXL1, IDH1, TP53,* and *NRAS*.

Safety toxicology

The safety, tolerability and toxicokinetic properties of luxeptinib were investigated via oral administration of luxeptinib up to the maximum feasible dose in rodents and dogs in pivotal 28-day GLP repeat-dose studies, which revealed dose-related increases in exposure but no drug-related adverse events through 28 days of twice-a-day dosing and a 14-day recovery period. No hematologic, cardiovascular or other organ toxicities were observed up to the highest dose of luxeptinib tested of 600 mg/kg administered orally in dogs. No effects were observed on any major organ including the central nervous or respiratory system in rodents who received single oral doses up to the highest dose of luxeptinib tested of 500 mg/kg. Luxeptinib did not inhibit the hERG potassium ion channel and a full cardiovascular study in dogs disclosed no effect on QTc or any other parameter of heart function. Metabolism studies documented that both CYP3A4 and CYP3A5 are involved in the metabolism of this drug.

In vivo activity of luxeptinib in a xenograft model of human AML

Pharmacodynamic studies of orally administered luxeptinib were performed using subcutaneously implanted MV4–11 FLT3-ITD xenograft model as part of toxicokinetic dose-ranging studies (**Fig. 5A** and **B**). This model does not predict activity of a drug on marrow-resident AML cells but does permit bridging of plasma drug levels to *in vitro* drug sensitivity studies. Mice were treated with luxeptinib or vehicle by oral gavage continuously, twice daily for 28 days. Inhibition of tumor growth was apparent at the lowest dose of 20 mg/kg/day, and efficacy increased with dose. Treatment completely suppressed tumor growth out to 120 days in 5 of 11 mice who received 100 mg/kg twice a day and in 10 of 11 mice treated with 300 mg/kg twice a day (**Fig. 5A**). All tumors that regrew in these two groups responded to reintroduction of a second 28-day treatment with luxeptinib even after reaching large tumor volumes (**Fig. 5B**). No weight loss or clinically apparent adverse events were observed even at doses of 300 mg/kg twice a day (**Fig. 5C**). At this dose level, the mean (± SEM) C_{min} plasma concentration was 1.03 ± 0.30 μmol/L. These results provide evidence of the tolerability of luxeptinib with this AML model.

Discussion

Luxeptinib was designed using structural information about FLT3 binding sites with the goal of simultaneously targeting both WT FLT3 and its clinically significant mutant forms. The studies reported here indicate this goal was achieved and disclose that luxeptinib also inhibits other kinases that participate in rescue pathways that support survival and mediate resistance to established FLT3 inhibitors. Screens using recombinant kinases pointed to a unique pattern of inhibition across the kinome with sub- or low nanomolar potency most apparent for the FLT3 cluster, but also including BTK, SRC, NTRK, STE/MAPK, and AURK clusters. A number of the additional targets captured by these clusters include pathways that have been implicated in disease pathogenesis and drug resistance in AML. These include targeting of PDGFRα in the rare FIP1L1-PDGFRα positive chronic eosinophilic leukemia form of AML (21) and of CSF1R to interrupt a paracrine signal between monocytes and AML cells (8, 9). In addition, mutation of *TP53* is a strong driver of drug resistance in both AML, including resistance to FLT3 inhibitors (6) and venetoclax (10, 22, 23). Targeting of NTRKs that become upregulated in the context of *TP53* mutation has been suggested to exhibit synthetic lethality in this setting of *TP53* mutation (10). Thus, luxeptinib offers the possibility of simultaneously inhibiting FLT3 and additional kinases that can impart resistance to a variety of other drugs through rescue pathways. The ability to impair the function of upstream kinases results in decreased activity of important downstream pathways including JAK/STAT5, MAPK, and ERK1/2.

Two important features became apparent when luxeptinib was compared with other FLT3 inhibitors: first, luxeptinib is a highly potent inhibitor of all forms of FLT3; and second, it retains the ability to kill a diverse set of patient derived AML samples, regardless of the FLT3 status, generally to a greater degree than the other agents. Information on how luxeptinib binds to FLT3 is not available, but it inhibited the activity of recombinant FLT-ITD at a 10-fold lower concentration than WT FLT3. Against other mutant forms of recombinant FLT3, luxeptinib had IC_{50} values in the range of 0.8 to 16 nmol/L and this range of potency was replicated in studies of the antiproliferative activity of luxeptinib on Ba/F3 cells engineered to express FLT3-ITD, FLT3-D835Y, or FLT3-ITD also containing the D835Y or F691 L point mutations. Luxeptinib was substantially more potent than quizartinib (4-fold), gilteritinib (53-fold), and crenolanib (70-fold) against FLT3-ITD in the Ba/F3 system and these differences were confirmed in the AML patient samples analyzed in the Beat AML project. Luxeptinib had IC_{50} values of <0.1 μmol/L for a large fraction of both WT and FLT3-ITD cases. The fraction of sensitive cells was less for quizartinib, gilteritinib and much lower for dovitinib, sorafenib, midostaurin, and crenolanib for the FLT3 WT cases, and for all these drugs, the fraction of cases with this degree of sensitivity was less in the FLT3-ITD cases.

WES data available from AML patient samples through the Beat AML project provided insight into the impact of disease-related gene mutations on the cytotoxicity of luxeptinib and related FLT3 inhibitors. Samples with either the FLT3-ITD variant or kinase domain mutations or both were more sensitive to luxeptinib than FLT3 WT samples. Mutations in *ASXL1, TP53, IDH2,* and *NRAS* did not associate with significantly higher mean IC_{50} values in pairwise comparisons. There was a trend toward greater sensitivity in samples containing the R132 mutation in *IDH1* although this did not reach statistical significance. Interestingly, and again perhaps reflecting the multi-cluster targeting capacity of luxeptinib, analysis of the RNA-seq data did not identify any individual genes, or a multigene expression

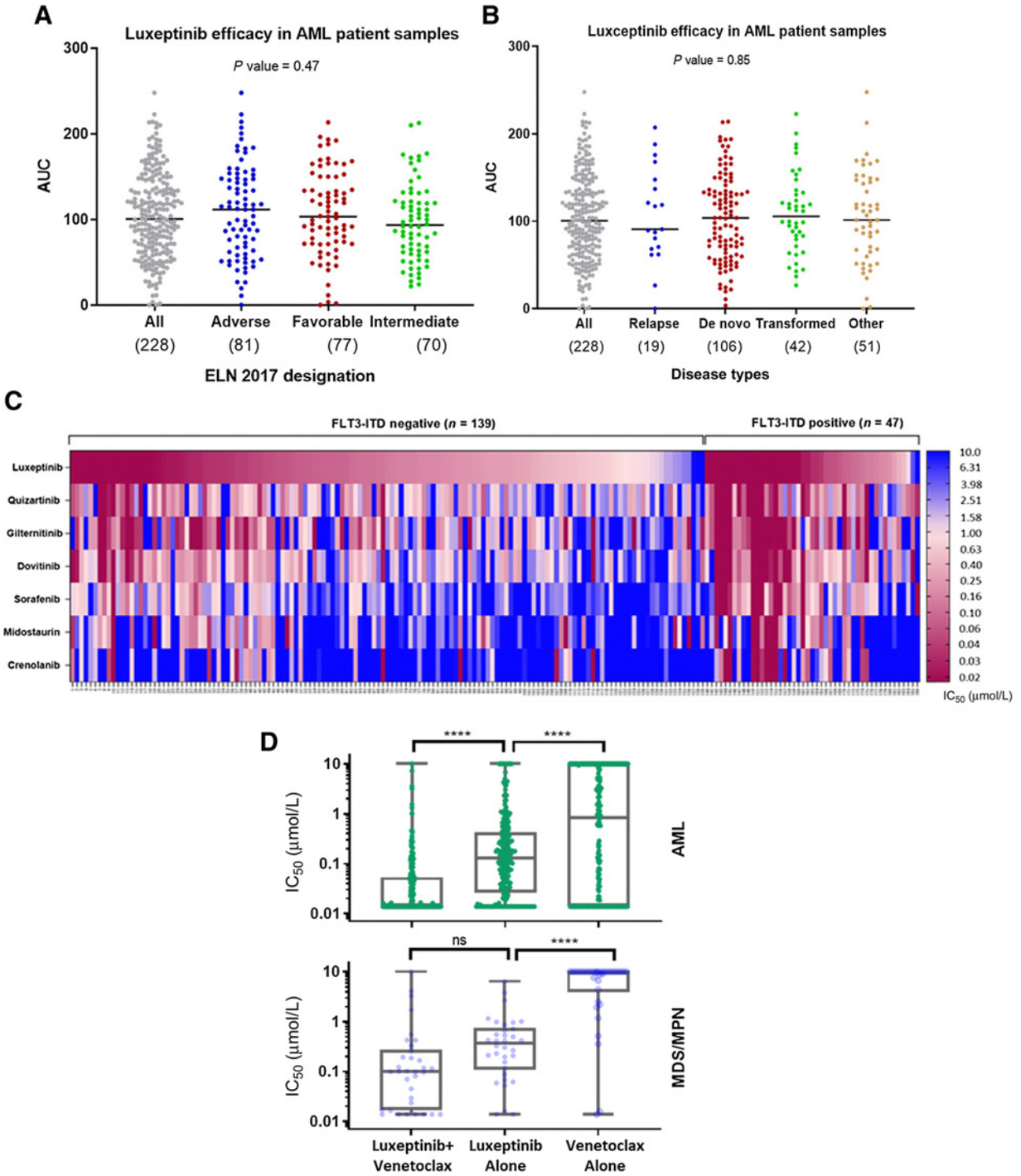
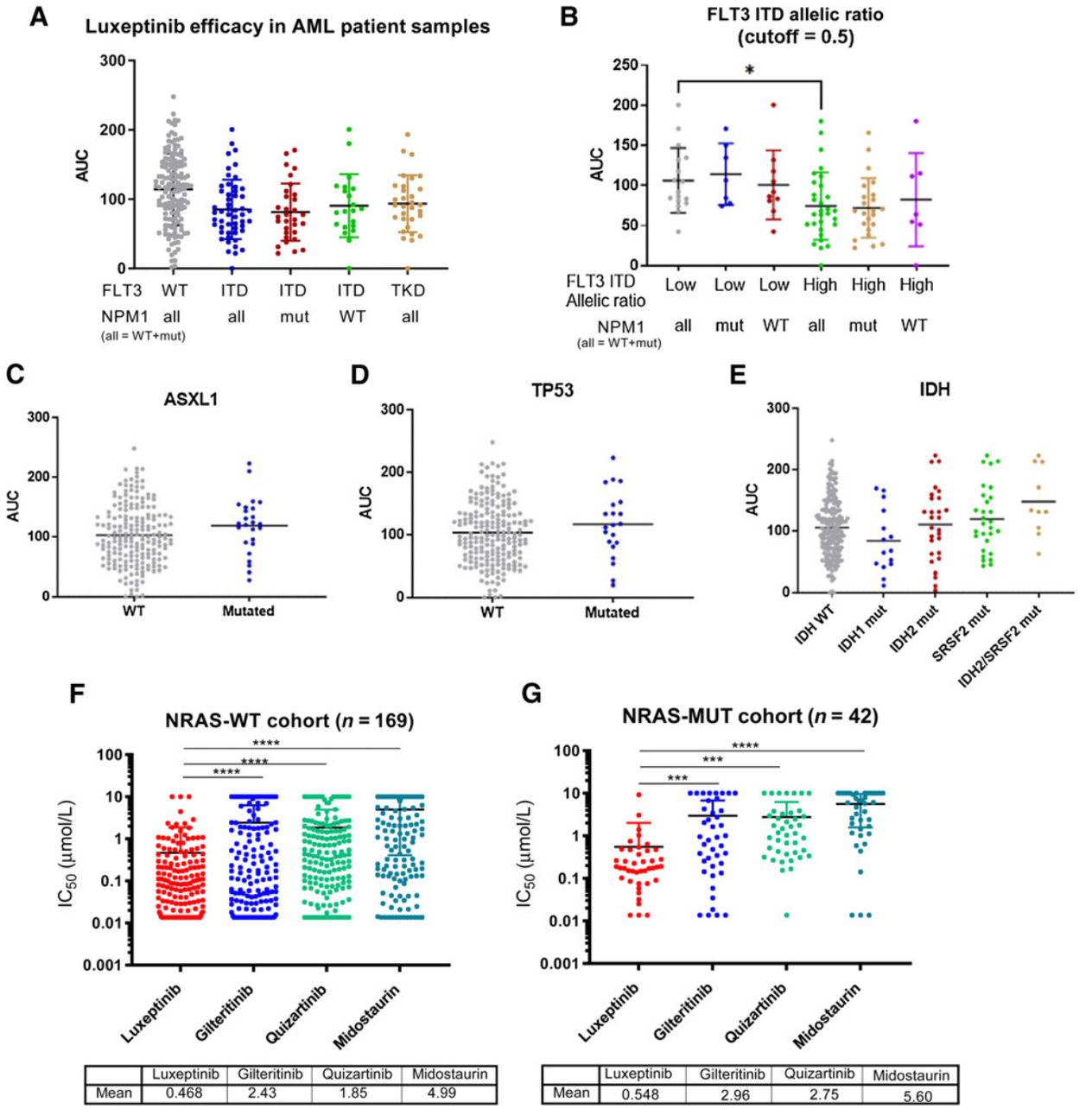


Figure 3.

Antiproliferative activity of luxeptinib on cells obtained from patients. Luxeptinib efficacy on subpopulations of AML patient samples according to ELN 2017 category (**A**) and type of disease (**B**). Horizontal line indicated median value; AUC is area under the concentration–survival curve. **C,** Heatmap depicting the effect of *FLT3*-ITD mutations on the IC_{50} values of luxeptinib and other FLT3 inhibitors tested against the same 186 patient samples; samples are grouped by *FLT3* mutational status. **D,** Inhibitory effect of luxeptinib and venetoclax alone or in combination on bone marrow and peripheral blood samples from patients with AML ($n = 232$) and MDS/MPN ($n = 34$). One-way analysis of variance and Student *t* test was used. ****, $P < 0.0001$; ns, not significant ($P > 0.05$).



	Luxeptinib	Gilteritinib	Quizartinib	Midostaurin
Mean	0.468	2.43	1.85	4.99

	Luxeptinib	Gilteritinib	Quizartinib	Midostaurin
Mean	0.548	2.96	2.75	5.60

Figure 4.

Comparison of the relative effect of mutations in selected genes cells detect in patient samples on luxeptinib (CG-806) and related FLT3 inhibitors. **A,** Impact of *FLT3* and *NPM1* mutations on sensitivity to luxeptinib as measured by area under the concentration–survival curve. **B,** Analysis of the effect of *FLT3-ITD* allelic ratio and *NPM1* mutation on sensitivity to luxeptinib. **C–E,** Effect of mutations in *ASXL1, TP53, IDH1, IDH2,* and *SRSF2* on IC_{50}. **F** and **G**, Distribution of IC_{50} values for luxeptinib, gilteritinib, quizartinib, and midostaurin in samples with WT (**F**) or mutant NRAS (**G**). Horizontal bar indicates median. Student *t* test was used. ****, $P < 0.0001$; ***, $P < 0.001$; *, $P < 0.05$; ns, not significant ($P > 0.05$).

profile, that engendered significant loss of sensitivity to luxeptinib. Further analysis of this data set is under way.

Given its broad application to hematologic malignancies, venetoclax is a strong candidate for partnering with luxeptinib in combination chemotherapy regimens. The data on the effectiveness of each agent alone and in combination from large number of AML and MDS/MPN patient samples, and the lack of dose-limiting overlapping clinical adverse events that has permitted venetoclax to be safely combined with several other drugs (20, 24), provide support for clinical testing of venetoclax in combination with luxeptinib.

In summary, luxeptinib is a distinctive kinase inhibitor. It inhibits WT FLT3 at nanomolar concentrations but is even more potent against FLT3-ITD and the other most clinically significant mutant forms. Importantly, rather than inhibiting just FLT3, luxeptinib targets

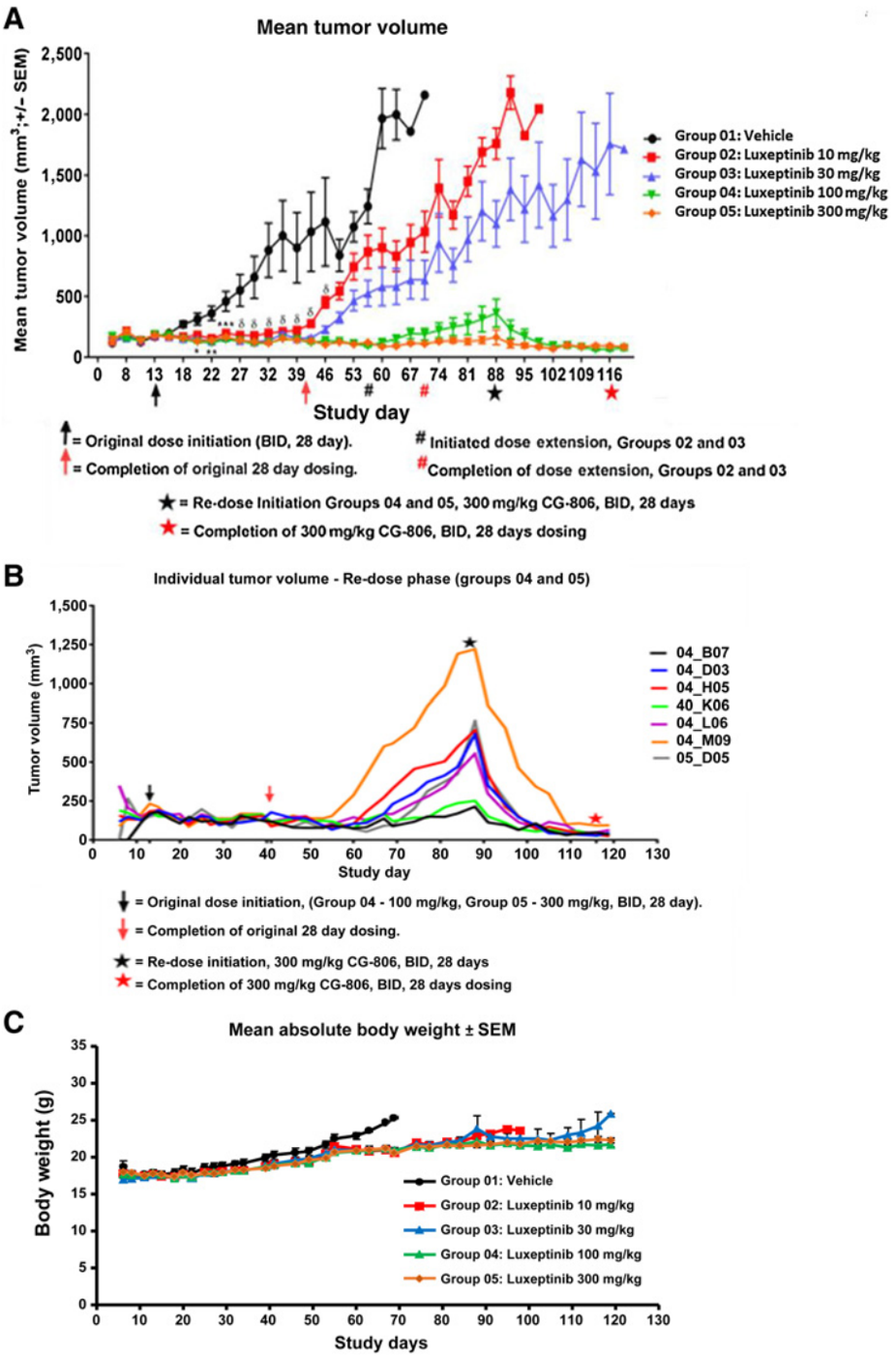


Figure 5.

Activity of luxeptinib (CG-806) in myeloid xenograft models. **A,** Dose-related inhibition of the growth of MV4–11 subcutaneous xenografts by luxeptinib given orally continuously (10–300 mg/kg twice a day, 8 mice per group) on a 28-day cycle; **B,** Response of individual recurrent MV4–11 tumors to re-treatment on a 28-day cycle with luxeptinib at 300 mg/kg twice a day. **C,** Absolute body weight (mean ± SEM) of mice in each group over the 120-day study period.

additional kinases that function in survival, support and resistance pathways. It was found to be active at concentrations of <0.1 μmol/L in a greater fraction of AML cases than other available FLT3 inhibitors. While the potency against both FLT3 and other kinases that function in this pathway may be clinically advantageous, it makes it difficult to attribute the therapeutic efficacy to the inhibition of any individual kinase. Additional studies are required to determine the extent to which cell death can be attributed to inhibition of any one target. Nevertheless, on the basis of its efficacy, luxeptinib was advanced to a phase I trial in patients with AML and MDS/MPN which established that doses of luxeptinib that yield plasma concentrations up to 1 μmol/L, and produce almost complete inhibition of FLT3 in a plasma inhibition assay, are generally well tolerated.

Authors' Disclosures

W.G. Rice reports other support from Aptose Biosciences Inc. outside the submitted work; in addition, W.G. Rice has a patent for Inventor on 4 patent applications related to the use of luxeptinib as an inhibitor of FLT3 and BTK to

treat certain hematologic malignancies pending. S.B. Howell reports personal fees from Aptose Biosciences Inc. during the conduct of the study; personal fees from Abeona Therapeutics outside the submitted work. H. Zhang reports employment at Aptose Biosciences Inc. N. Rastgoo reports other support from Aptose Biosciences Inc. during the conduct of the study; other support from Aptose Biosciences Inc. outside the submitted work; in addition, N. Rastgoo has a patent for Aptose Biosciences Inc. licensed. S.E. Kurtz reports other support from OHSU/Aptose Biosciences Inc. during the conduct of the study. P. Lo reports other support from Aptose Biosciences Inc. during the conduct of the study; other support from Agios, Array BioPharma, AstraZeneca, Constellation Pharmaceuticals, Genentech, Gilead Sciences, Incyte, Janssen, Petra, Seattle Genetics, Syros, Takeda; and other support from Tolero outside the submitted work. B. Wilmot reports grants from NIH during the conduct of the study. S.K. McWeeney reports grants from NIH NCI U54CA224019; and grants from NIH NCATS UL1TR002369 during the conduct of the study. B.J. Druker reports grants from NIH NCI U01 during the conduct of the study; personal fees from Aileron Therapeutics, Aptose Biosciences Inc., Iterion Therapeutics, Blueprint Medicines, Cepheid, GRAIL, Enliven Therapeutics, Nemucore Medical Innovations, Novartis, Recludix Pharma, VB Therapeutics, Vincerx Pharma, Vivid Biosciences, Beat AML LLC, CureOne, Gilead Sciences, ICON (formerly MolecularMD), Monojul, Pfizer, Adela Bio, RUNX1 Research Program, Celgene; personal fees and nonfinancial support from Amgen, Burroughs Wellcome Fund; and personal fees from AstraZeneca outside the submitted work; in addition, B.J. Druker has a patent for Treatment of Gastrointestinal Stromal Tumors issued, licensed, and with royalties paid from Novartis, a patent for Detection of Gleevec Resistant Mutations Detection of Gleevec resistance issued to MolecularMD (acquired by ICON), a patent for Methods, Apparatuses, and Systems for Detecting and Quantifying Phosphoproteins issued, a patent for Methods, Systems, and Apparatuses for Quantitative Analysis of Heterogeneous Biomarker Distribution issued, and a patent for Methods, Systems, and Apparatuses for Quantitative Analysis of Heterogeneous Biomarker Distribution issued. J.W. Tyner reports other support from Aptose Biosciences Inc. during the conduct of the study; other support from Agios, Array BioPharma, AstraZeneca, Constellation Pharmaceuticals, Genentech, Gilead Sciences, Incyte, Janssen, Petra, Seattle Genetics, Syros, Takeda; and other support from Tolero outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

W.G. Rice: Conceptualization, resources, supervision, funding acquisition, investigation, writing–original draft, project administration, writing–review and editing. **S.B. Howell:** Conceptualization, formal analysis, investigation, writing–original draft, writing–review and editing. **H. Zhang:** Formal analysis, investigation, writing–original draft, writing–review and editing. **N. Rastgoo:** Investigation. **A. Local:** Investigation. **S.E. Kurtz:** Conceptualization, formal analysis, investigation. **P. Lo:** Formal analysis, investigation. **D. Bottomly:** Data curation, software, formal analysis, investigation. **B. Wilmot:** Formal analysis, investigation. **S.K. McWeeney:** Formal analysis, investigation. **B.J. Druker:** Supervision, funding acquisition, project administration, writing–review and editing. **J.W. Tyner:** Conceptualization, data curation, formal analysis, investigation, writing–review and editing.

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