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ORIGINAL RESEARCH

Anti-Tumor Effects of Gilteritinib on *FLT3* Mutations: Insights into Resistance Mechanisms in Ba/F3 Cell Models

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Background: The efficacy of Fms-like tyrosine kinase 3 (FLT3) inhibitors has been well established against acute myeloid leukemia (AML) with *FLT3* internal tandem duplication (*FLT3*-ITD) mutations. However, comparative data on the available inhibitors are limited, and drug resistance remains a major concern.

Methods: This study examined the inhibitory effects of gilteritinib, quizartinib and midostaurin on Ba/F3 cells with *FLT3*-ITD mutations, or point mutations in the tyrosine kinase domain (TKD-PM) or juxtamembrane domain (JMD-PM), both in vitro and in vivo. Quizartinib and midostaurin were selected as comparators due to their clinical relevance in the AML setting and differing mechanisms of action.

Results: Gilteritinib showed similar or superior growth inhibition against the majority of *FLT3*-TKD-PM or *FLT3*-JMD-PM cells compared to *FLT3*-ITD-mutant cells. In contrast, the inhibitory effects of quizartinib were reduced on cells with most types of *FLT3*-TKD-PM, and the inhibitory effects of midostaurin were attenuated on cells with *FLT3*-TKD-PM N676K. Gilteritinib also effectively suppressed FLT3 autophosphorylation and phosphorylation of signal transducer and activator of transcription 5 (STAT5), AKT and extracellular signal-regulated kinase (ERK) in *FLT3*-TKD-PM cells, while quizartinib showed a reduced inhibitory effect on FLT3 autophosphorylation and phosphorylation of downstream signaling molecules in *FLT3*-TKD-PM cells. In mice xenografted with Ba/F3 cells expressing *FLT3*-ITD mutations or *FLT3*-TKD-PM, gilteritinib showed a potent antitumor effect, whereas the antitumor effect of quizartinib was significantly diminished in the *FLT3*-TKD-PM xenograft model.

Conclusion: These findings highlight the potent efficacy of gilteritinib against a wide range of *FLT3* mutations, including TKD-PM and JMD-PM, as well as those associated with resistance to quizartinib or midostaurin. This comparative analysis underscores the need for tailored therapeutic strategies in AML treatment, emphasizing the clinical significance of gilteritinib in overcoming drug resistance.

Keywords: acute myeloid leukemia, gilteritinib, *FLT3*-TKD, *FLT3*-JMD, drug resistance, xenograft mouse models

Introduction

Acute myeloid leukemia (AML) is a complex, evolving disease involving multiple somatic driver mutations, with patients often harboring more than one mutation.[1] Indeed, Papaemmanuil et al identified approximately 5300 driver mutations across 76 genes or genomic regions, with 86% of patients studied having at least two driver mutations.[1]

Activating mutations in FMS-like tyrosine kinase 3 (FLT3) are common in AML.[1–3] Internal tandem duplication (*FLT3*-ITD) mutations are found in ~20% to 25% of patients, while point mutations in the tyrosine kinase domain (*FLT3*-TKD-PM) occur in 5–10% of patients, particularly at the activation loop residue, D835.[1–3] Patients with *FLT3*-ITD mutations, are associated with poor prognosis including short duration of remission and increased risk of relapse.[3,4] However, the prognostic value of *FLT3*-TKD-PM remains unknown.[4] In addition to *FLT3*-ITD mutations and *FLT3*-TKD-PM, point mutations in the juxtamembrane domain of FLT3 (*FLT3*-JMD-PM) have been identified in 1–2.4% of patients with AML.[5–8] These mutations also exhibit oncogenic phenotypes and resistance to apoptotic cell death.[6]

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In the last decade, there have been significant advances in AML treatment, with three FLT3 inhibitors approved for the treatment of *FLT3*-mutated AML, namely, midostaurin,[9] gilteritinib,[10] and most recently, quizartinib.[11] Midostaurin is a first-generation, type I FLT3 inhibitor approved for the treatment of adult patients with newly diagnosed AML who are *FLT3* mutation-positive, in combination with standard cytarabine and daunorubicin induction and cytarabine consolidation, or as maintenance monotherapy in patients who achieve a complete response.[12,13] Gilteritinib and quizartinib are second-generation type I and type II FLT3 inhibitors, respectively.[12,13] Gilteritinib is approved for the treatment of adult patients with relapsed or refractory AML who are *FLT3* mutation-positive.[10,14] Quizartinib is approved for the treatment of adult patients with newly diagnosed AML with *FLT3*-ITD mutations and relapsed/refractory AML (Japan) with *FLT3*-ITD mutations.[11,15,16] Despite these advancements, there remain challenges in the clinical management of AML, including resistance to FLT3 inhibitors and the need for combination therapies or next-generation inhibitors to improve patient outcomes. Recent studies have focused on understanding the resistance mechanisms and exploring novel therapeutic strategies to overcome these hurdles. Additionally, ongoing clinical trials are investigating the efficacy of combining FLT3 inhibitors with other targeted therapies and immunotherapies, which may offer new hope for patients with *FLT3*-mutated AML.

Previous in vitro and in vivo studies have demonstrated the inhibitory effect of gilteritinib on the growth of cells with both *FLT3*-ITD mutations and *FLT3*-TKD-PM, including in the presence of activating and tyrosine kinase inhibitor-resistant mutations.[3,17–19] Type II inhibitors like quizartinib are ineffective against certain active conformations of *FLT3* mutations, while gilteritinib, a type I inhibitor, binds effectively to both active and inactive conformations, enabling broader efficacy. This difference in binding specificity or inhibition of downstream signaling may contribute to the broader efficacy of gilteritinib, covering a wide spectrum of *FLT3* mutations, including those resistant to other FLT3 inhibitors like quizartinib and midostaurin. Further, FLT3 ligand (FL) is associated with resistance to FLT3 inhibitors in AML harboring both *FLT3*-wild type or *FLT3* mutations.[20,21] Unlike other FLT3 inhibitors, gilteritinib was shown to have inhibitory and apoptotic activity in vitro even in the presence of FL overexpression.[21] This broader efficacy of gilteritinib represents a significant advancement in addressing a critical challenge in AML resistant to existing FLT3 inhibitors like quizartinib and midostaurin. Therefore, the efficacy of gilteritinib against various *FLT3* mutations may offer a more reliable treatment option for patients with drug-resistant AML, potentially reducing relapse rates and improving survival.

The objective of the present study was to examine the inhibitory effects of gilteritinib, quizartinib and midostaurin on cells harboring a *FLT3*-ITD mutations, *FLT3*-TKD-PM, or *FLT3*-JMD-PM. This research comprehensively evaluates the efficacy of gilteritinib against a wide range of *FLT3* mutations, including those that confer resistance to other FLT3 inhibitors such as quizartinib and midostaurin.

Materials and Methods

We conducted in vitro and in vivo studies using mutant type FLT3-expressing Ba/F3 cells to assess the inhibitory effect of gilteritinib on cells with *FLT3*-TKD-PM or *FLT3*-JMD-PM, compared with cells with *FLT3*-ITD mutations as a reference standard. The inhibitory effects of quizartinib and midostaurin were also investigated as comparators.

Compounds and Plasmids

Gilteritinib hemifumarate (gilteritinib) was synthesized by Astellas Pharma Inc. (Tokyo, Japan). Quizartinib and midostaurin for in vitro study were purchased from Selleck Chemicals (Houston, Texas, United States of America [USA]), and LC Laboratories (Woburn, Massachusetts, USA), respectively. Quizartinib dihydrochloride for in vivo study was synthesized by Sumika Technoservice Co (Hyogo, Japan). For in vitro use, all compounds were dissolved in dimethyl sulfoxide (DMSO) and frozen. For in vivo studies, gilteritinib was dissolved in 0.5% methyl cellulose 400 solution (0.5% MC) while quizartinib was dissolved in 22% 2-hydroxypropyl-β-cyclodextrin (22% HP-β-CD).

FLT3-ITD mutations, *FLT3*-TKD-PM and *FLT3*-JMD-PM were N-terminally tagged with FLAG and cloned into the pMXs-Puro retroviral vector (Cell Biolabs, San Diego, California, USA). Human *FLT3* (NM_004119.2) was used as

a reference sequence. The ITD sequence of *FLT3*-ITD was derived from MOLM-13 cells.[22] *FLT3*-TKD-PM *and FLT3*-JMD-PM constructed in this study are shown in Table 1.

Anti-phospho-STAT5 (Y694) were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA), while antibodies against phospho-FLT3 (Y591), total FLT3, total STAT5, phospho-AKT (S473), total AKT, phospho-ERK1/2 (T202/Y204), total ERK and β-actin were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA).

Cell Lines and Cell Culture

The cell lines used in this study were not authenticated in our laboratory but were purchased from the providers of authenticated cell lines and stored at early passages in a central cell bank at Astellas Pharma Inc. Experiments were conducted using low-passage cultures of these stocks that were confirmed negative for mycoplasma contamination.

The mouse pro-B cell line Ba/F3 was purchased from RIKEN Cell Bank (Ibaraki, Japan) and cultured at 37°C in 5% CO_2 humidified atmosphere in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 10 ng/mL mouse interleukin-3 (IL-3). Platinum-E cells were provided by Dr. Toshio Kitamura (University of Tokyo, Japan) and cultured at 37°C in 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin. Ba/F3 cells expressing mutated FLT3 were generated by Astellas Pharma Inc. as follows:

Table 1 Inhibitory Effect of Gilteritinib, Quizartinib and Midostaurin on the Growth of Ba/F3 Cells Expressing Mutated *FLT3*

***FLT3* mutation**	**Domain[a]**	**IC_{50} nM (95% CI)**		
		Gilteritinib	**Quizartinib**	**Midostaurin**
ITD	JMD	4.5 (3.0–6.8)	0.51 (0.38–0.68)	4.7 (3.4–6.6)
D835V	TKD	0.60 (0.22–1.6)	13 (6.9–26)	0.80 (0.43–1.5)
D835E	TKD	0.96 (0.45–2.0)	0.88 (0.51–1.5)	2.5 (1.6–3.8)
D835A	TKD	0.97 (0.43–2.2)	1.1 (0.45–2.8)	2.4 (1.3–4.5)
D835N	TKD	1.0 (0.43–2.5)	1.2 (0.76–1.9)	4.2 (2.2–7.9)
D835G	TKD	1.4 (0.79–2.5)	1.2 (0.80–1.7)	1.7 (0.84–3.5)
D835L+K	TKD	2.6 (1.9–3.7)	25 (18–34)	1.2 (1.1–1.3)
D835del	TKD	0.87 (0.44–1.7)	12 (8.6–18)	1.1 (0.70–1.6)
I836del	TKD	3.5 (2.2–5.6)	0.71 (0.30–1.7)	2.4 (1.9–3.2)
I836L+D	TKD	1.7 (0.79–3.9)	1.2 (0.37–4.0)	2.3 (1.4–3.7)
I836S	TKD	4.0 (2.0–8.0)	0.40 (0.23–0.72)	1.4 (0.62–3.0)
I836T	TKD	3.9 (3.6–4.2)	0.31 (0.23–0.43)	2.3 (1.2–4.4)
N841Y	TKD	0.85 (0.70–1.0)	1.3 (1.1–1.5)	2.5 (2.0–3.2)
R845S	TKD	4.8 (3.6–6.5)	0.87 (0.62–1.2)	3.4 (2.5–4.7)
N676K	TKD	0.99 (0.59–1.7)	3.7 (2.6–5.4)	15 (10–23)
Y572C	JMD	1.1 (0.42–2.9)	0.15 (0.083–0.28)	0.91 (0.61–1.3)
F590G/Y591D	JMD	5.0 (1.8–14)	0.18 (0.12–0.26)	1.5 (0.99–2.2)
F590 D593delinsLY	JMD	4.9 (1.6–15)	0.23 (0.16–0.34)	1.6 (0.87–3.0)

(*Continued*)

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Table 1 (Continued).

FLT3 mutation	Domain[a]	IC_{50} nM (95% CI)		
		Gilteritinib	Quizartinib	Midostaurin
V592G	JMD	4.8 (2.0–11)	0.21 (0.13–0.36)	1.5 (0.96–2.3)
V592A	JMD	8.6 (1.6–47)	0.39 (0.28–0.53)	3.4 (2.7–4.3)
D593del	JMD	5.8 (1.9–18)	0.39 (0.33–0.46)	2.9 (1.7–4.8)
F594L	JMD	5.5 (1.7–18)	0.35 (0.20–0.64)	1.8 (0.73–4.5)
V579A	JMD	2.5 (0.86–7.3)	0.41 (0.32–0.53)	2.7 (1.5–5.0)
E598/Y599del	JMD	1.0 (0.40–2.5)	0.36 (0.31–0.43)	2.2 (1.1–4.5)
K663R	TKD	1.5 (0.33–7.0)	0.43 (0.27–0.70)	2.6 (1.5–4.5)
K663Q	TKD	0.98 (0.40–2.4)	0.46 (0.26–0.83)	3.0 (1.2–7.3)
R834Q	TKD	2.4 (0.62–9.1)	0.55 (0.35–0.88)	1.3 (1.0–1.6)
D839G	TKD	0.56 (0.14–2.2)	0.84 (0.57–1.3)	1.5 (0.63–3.8)
S840insGS	TKD	1.2 (0.28–5.6)	0.94 (0.40–2.2)	1.1 (0.36–3.3)
N841I	TKD	0.77 (0.22–2.7)	1.3 (0.89–1.9)	4.4 (2.2–8.7)
Y842C	TKD	1.4 (0.38–5.5)	3.5 (1.8–6.8)	0.89 (0.36–2.2)
I867S	TKD	1.8 (0.53–6.3)	0.37 (0.25–0.55)	3.4 (1.5–7.8)

Notes: [a]1. Reindl C, Bagrintseva K, Vempati S, Schnittger S, Ellwart JW, Wenig K, Hopfner KP, Hiddemann W, Spiekermann K. Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML. *Blood*. 2006; 107: 3700–7. doi: 10.1182/blood-2005-06-2596. 2. The Clinical Knowledgebase. The Jackson Laboratory. Available at: Gene Detail (jax.org 1). Accessed February 2024.
Abbreviations: CI, confidence interval; FLT3, Fms-like tyrosine kinase 3; IC_{50}, half-maximal inhibitory concentration.

pMXs-Puro retroviral vectors (Cell Biolabs) containing each *FLT3* mutant vector was transfected into Platinum-E cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to produce the virus; Ba/F3 cells were then infected with viral supernatant containing each *FLT3* mutant vector; Ba/F3 cells expressing mutated FLT3 were selected using puromycin at 1.0 μg/mL and subsequently cultured in the mouse IL-3-deprived medium.

Cell Viability Assay

Ba/F3 cells expressing mutated FLT3 were seeded at 1×10^3 cells/well in 96-well plates. After culturing overnight, cells were treated with gilteritinib, quizartinib or midostaurin at 0–1000 nM (10 points, approximately a 3-fold serial dilution) for 2 days. Cell viability was measured with the CellTiter-Glo® 2.0 Assay (Promega Corporation, Madison, Wisconsin, USA) and measurement of luciferase activity performed with SpectraMax Paradigm (Molecular Devices, Sunnyvale, California, USA). Data were analyzed using GraphPad PRISM v7.0.5 (GraphPad Software, Inc., San Diego, California, USA). Cell viability was calculated by defining untreated cells and medium control wells as 100% and 0%, respectively, and the half-maximal inhibitory concentration (IC_{50}) value calculated using Sigmoid-Emax model nonlinear regression analysis. Geometric means and 95% confidence intervals (CIs) of IC_{50} values were determined from three or six independent experiments performed in duplicate.

Western Blot Analysis

Cells were seeded at 2×10^6 cells/well in 6-well plates and treated with gilteritinib or quizartinib for 2 hours. Following treatment, cells were harvested and lysed with RIPA buffer (Thermo Fisher Scientific) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The protein concentrations of each lysate were measured by bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). The cell lysates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with equal amount of protein and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with Blocking One (Nacalai Tesque, Kyoto, Japan), the membranes were incubated with primary antibodies at room temperature overnight. After washing with Tris-buffered saline with 0.1% Tween® (TBS-T, Thermo Fisher Scientific), each membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Proteins of interest were detected using Western Lightning Plus-ECL (PerkinElmer, Waltham, Massachusetts, USA) or Western Lightning Ultra (PerkinElmer) with LAS-4000 (GE Healthcare, Chicago, Illinois, USA).

Animals

Four-week-old male nude mice (CAnN.Cg-Foxn1nu/CrlCrlj[nu/nu]) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and maintained on a standard diet and water throughout the experiments under specific pathogen-free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc., Tsukuba Research Center, which is accredited by AAALAC International.

In vivo Evaluation Using Xenograft Mouse Model

Mutant type *FLT3*-expressing Ba/F3 cells were suspended in phosphate buffered saline (PBS) and then mixed with an equivalent volume of Matrigel (BD Biosciences). Each mouse was inoculated subcutaneously with 3×10^6 cells in the right flank. Treatment was started after the tumor volume reached 100–300 mm^3 through oral administration of each compound once daily. Tumor size and body weight were measured using a caliper and an electronic balance, respectively. Tumor volume was calculated by the formula: length × $width^2$ × 0.5.

Statistical Analysis

For in vivo studies, tumor volumes were compared between gilteritinib-treated and quizartinib-treated groups using Student's *t*-test, with a p-value of <0.05 considered statistically significant. Microsoft Excel (Microsoft) and GraphPad PRISM v8.0.2 (GraphPad Software Inc., San Diego, California, USA) were used for data processing.

Results

Cell Viability Assays

The inhibitory effect of gilteritinib on the growth of Ba/F3 cells expressing *FLT3*-ITD mutations, as measured by IC_{50}, was 4.5 nM (95% CI 3.0–6.8 nM; Table 1). Similar, or more potent, inhibitory effects were seen on the growth of Ba/F3 cells expressing most types of *FLT3*-TKD-PM (Table 1). For cells expressing *FLT3*-JMD-PM Y572C, F590G/Y591D, V592G, V592A, F594L and V579A, gilteritinib showed similar inhibitory effects compared to its effect on cells expressing *FLT3*-ITD mutations (Table 1).

In contrast, the inhibitory effects of quizartinib on the growth of Ba/F3 cells expressing D835V, D835L+K, D835del, N676K or Y842C *FLT3*-TKD-PM were reduced by more than 6-fold compared with its effect on cells with *FLT3*-ITD mutations (Table 1). The growth inhibitory effect of midostaurin on Ba/F3 cells expressing *FLT3*-TKD-PM N676K mutations was reduced by approximately 3-fold compared with its effect on cells expressing *FLT3*-ITD mutations (Table 1). However, the effect of midostaurin on Ba/F3 cell growth was generally unaffected by any of the other mutations evaluated (Table 1).

Given that midostaurin showed no significant difference in activity against cells with *FLT3*-JMD-PM compared with *FLT3*-ITD mutations, and the only difference observed in its activity was for cells carrying *FLT3*-TKD-PM N676K, we decided to focus our Western blot analysis and in vivo studies on the activity of gilteritinib and quizartinib against *FLT3*-TKD-PM compared with *FLT3*-ITD mutations.

Western Blot Analysis

The inhibitory effects of gilteritinib and quizartinib on FLT3 and its downstream signaling molecules were investigated in Ba/F3 cells expressing *FLT3*-ITD mutations and Ba/F3 cells expressing *FLT3*-TKD-PM D835V, D835del, D835L+K, Y842C and N676K. Both gilteritinib and quizartinib showed an inhibitory effect on FLT3 autophosphorylation in Ba/F3 cells expressing *FLT3*-ITD mutations (Figure 1). Gilteritinib also showed an inhibitory effect on FLT3 autophosphorylation in Ba/F3 cells expressing each of the *FLT3*-TKD-PMs examined, equivalent to its effect on Ba/F3 cells expressing *FLT3*-ITD mutations (Figure 1). In contrast, the inhibitory effect of quizartinib on FLT3 autophosphorylation in Ba/F3 cells expressing these *FLT3*-TKD-PMs was reduced compared with its effect on Ba/F3 cells expressing *FLT3*-ITD mutations (Figure 1).

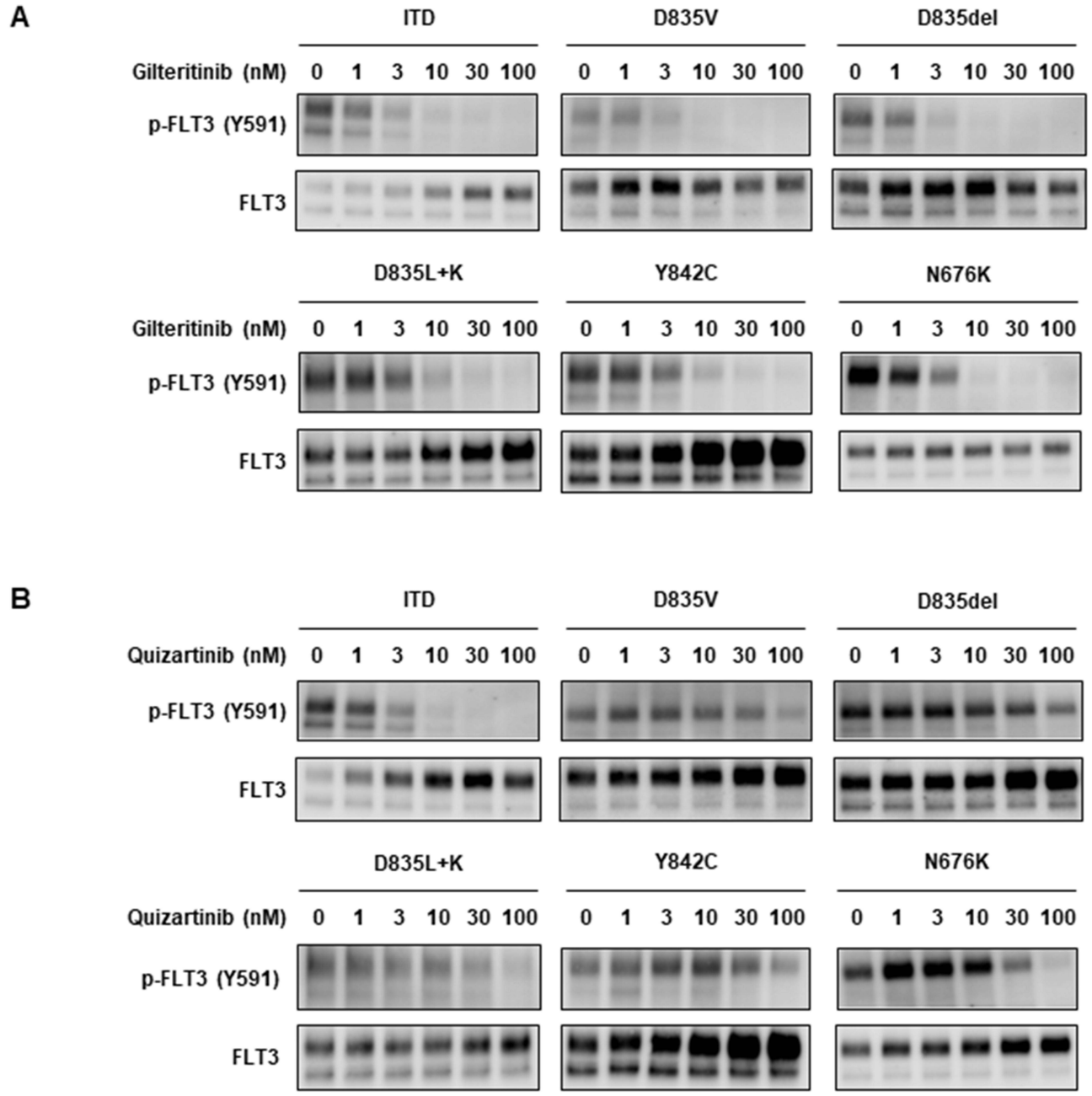

Figure 1 Inhibitory effect of gilteritinib (**A**) and quizartinib (**B**) on phosphorylated or total FLT3 in Ba/F3 cells expressing *FLT3*-ITD, *FLT3*-D835V, *FLT3*-D835del, *FLT3*-D835L+K, *FLT3*-Y842C or *FLT3*-N676K.

Note: Mutant type FLT3-expressing Ba/F3 cells were treated with gilteritinib or quizartinib for 2 hours.

Abbreviations: FLT3, Fms-like tyrosine kinase 3; ITD, internal tandem duplication; p, phosphorylated.

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Similarly, both gilteritinib and quizartinib suppressed phosphorylation of STAT5, AKT and ERK in *FLT3*-ITD cells (Figures 2–4). Regarding STAT5, AKT and ERK phosphorylation in cells with *FLT3*-TKD-PM, gilteritinib showed a more potent inhibitory effect on STAT5 phosphorylation in Ba/F3 cells expressing D835V, D835del and N676K compared with *FLT3*-ITD cells (Figures 2–4). On the other hand, the inhibitory effect of quizartinib on STAT5 phosphorylation in cells expressing D835V, D835del and D835L+K was attenuated compared with its inhibitory effect in *FLT3*-ITD cells (Figure 2). Regarding AKT phosphorylation in cells with *FLT3*-TKD-PM, gilteritinib showed an equivalent inhibitory effect to that observed in *FLT*-ITD cells, while the inhibitory effects of quizartinib in cells with all *FLT3*-TKD-PMs tested tended to be attenuated compared with that observed in *FLT3*-ITD cells (Figure 3). Gilteritinib

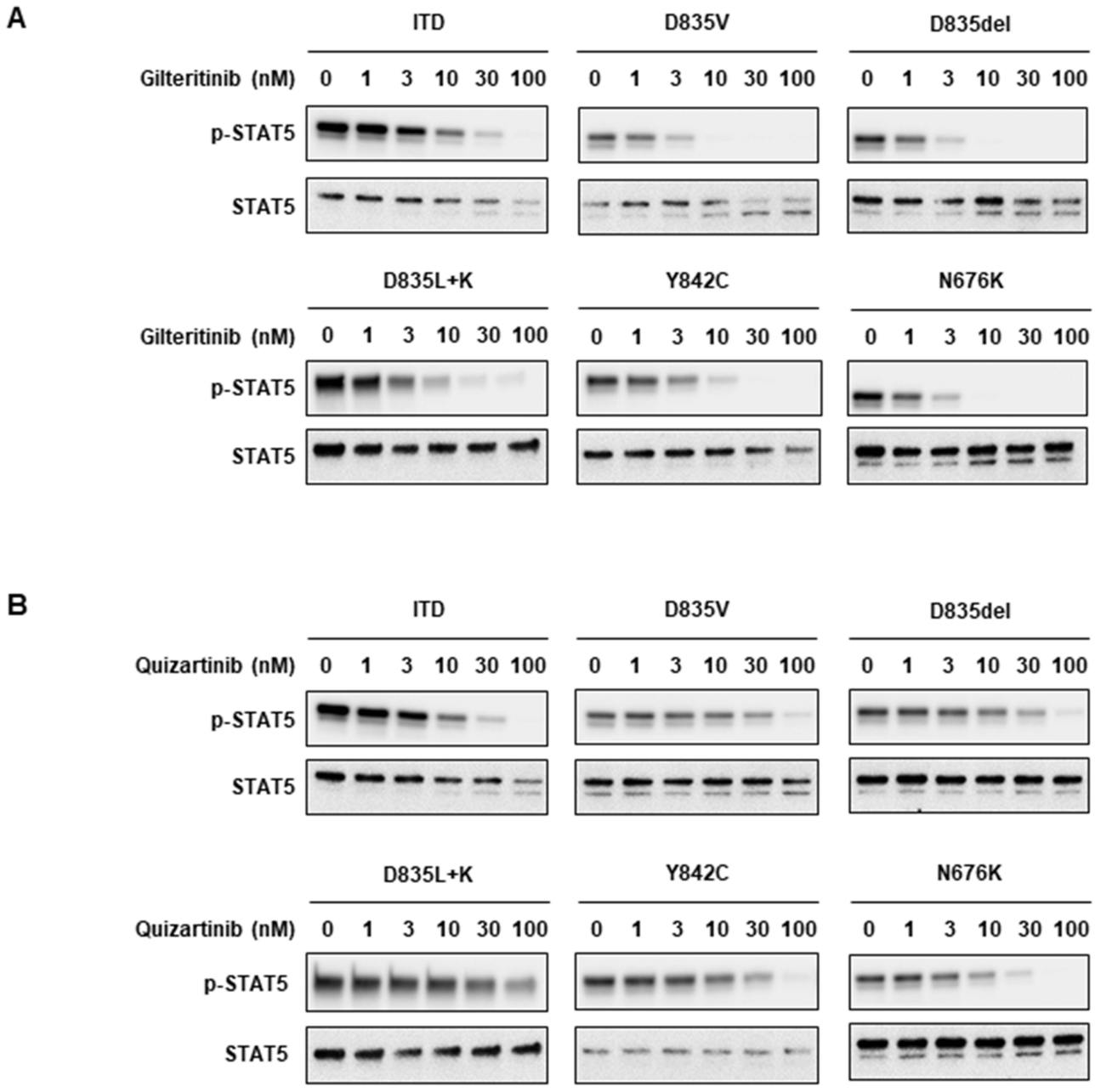

Figure 2 Inhibitory effect of gilteritinib (**A**) and quizartinib (**B**) on phosphorylated or total STAT5 in Ba/F3 cells expressing *FLT3*-ITD, *FLT3*-D835V, *FLT3*-D835del, *FLT3*-D835L+K, *FLT3*-Y842C or *FLT3*-N676K.

Note: Mutant type FLT3-expressing Ba/F3 cells were treated with gilteritinib or quizartinib for 2 hours.

Abbreviations: FLT3, Fms-like tyrosine kinase 3; ITD, internal tandem duplication; p, phosphorylated; STAT, signal transducer and activator of transcription.

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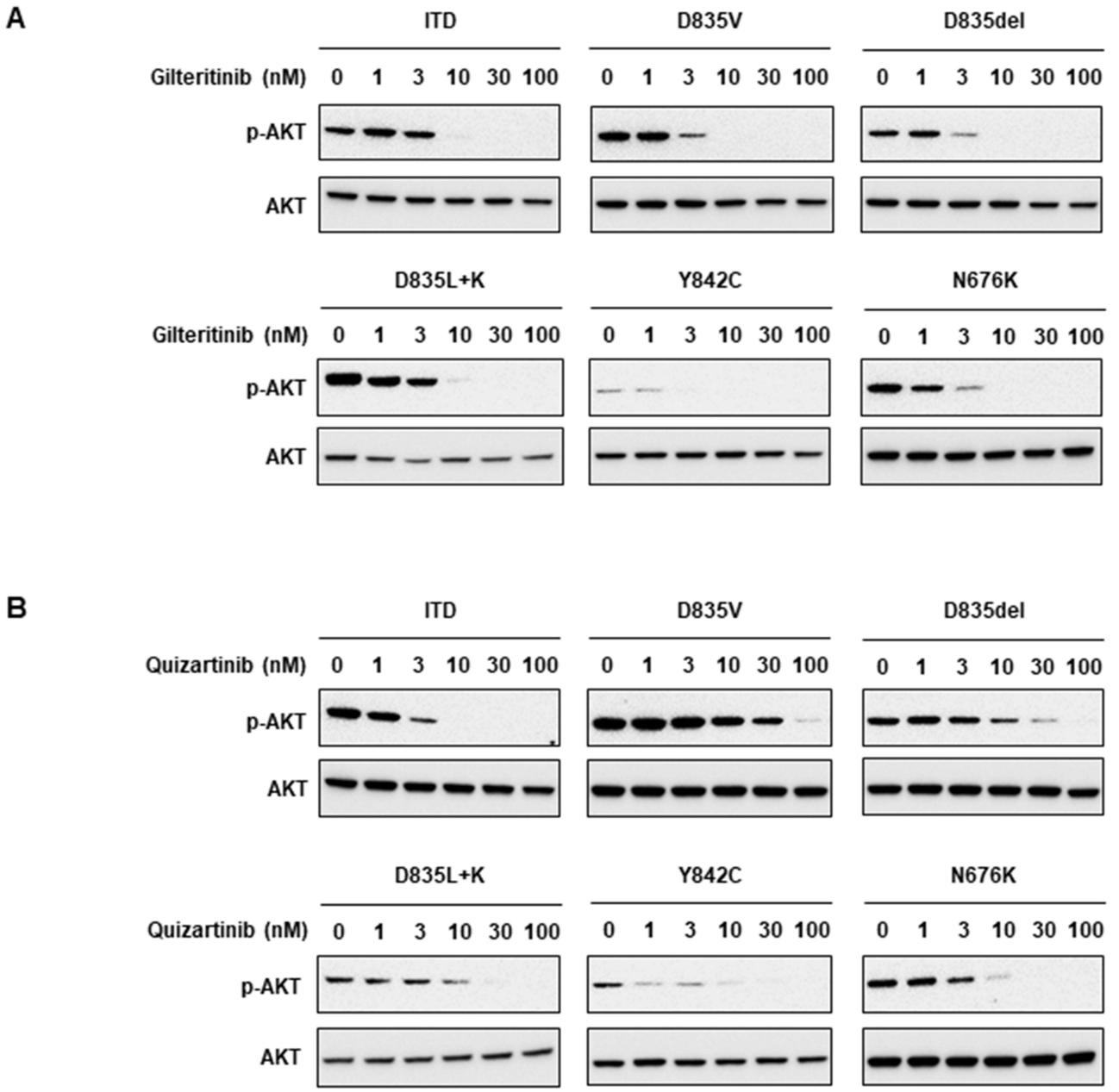

Figure 3 Inhibitory effect of gilteritinib (**A**) and quizartinib (**B**) on phosphorylated or total AKT in Ba/F3 cells expressing *FLT3*-ITD, *FLT3*-D835V, *FLT3*-D835del, *FLT3*-D835L+K, *FLT3*-Y842C or *FLT3*-N676K.
Note: Mutant type FLT3-expressing Ba/F3 cells were treated with gilteritinib or quizartinib for 2 hours.
Abbreviations: FLT3, Fms-like tyrosine kinase 3; ITD, internal tandem duplication; p, phosphorylated.

also showed an equivalent inhibitory effect on ERK phosphorylation in cells with *FLT3*-TKD-PM versus *FLT3*-ITD mutations, while the inhibitory effects of quizartinib on ERK phosphorylation in cells with *FLT3*-TKD-PMs were attenuated versus *FLT3*-ITD cells (Figure 4).

Antitumor Activity and Body Weight Change in Mutant FLT3-Expressing Ba/F3 Xenografted Mouse Model

Once-daily oral administration of 30 mg/kg gilteritinib or 10 mg/kg quizartinib showed a potent and equivalent antitumor effect in an *FLT3*-ITD mutant xenograft model (Figure 5). Gilteritinib also showed a strong antitumor effect in mice xenografted with Ba/F3 cells expressing *FLT3*-TKD-PM D835V, D835del, D835L+K, N676K and Y842C, whereas the

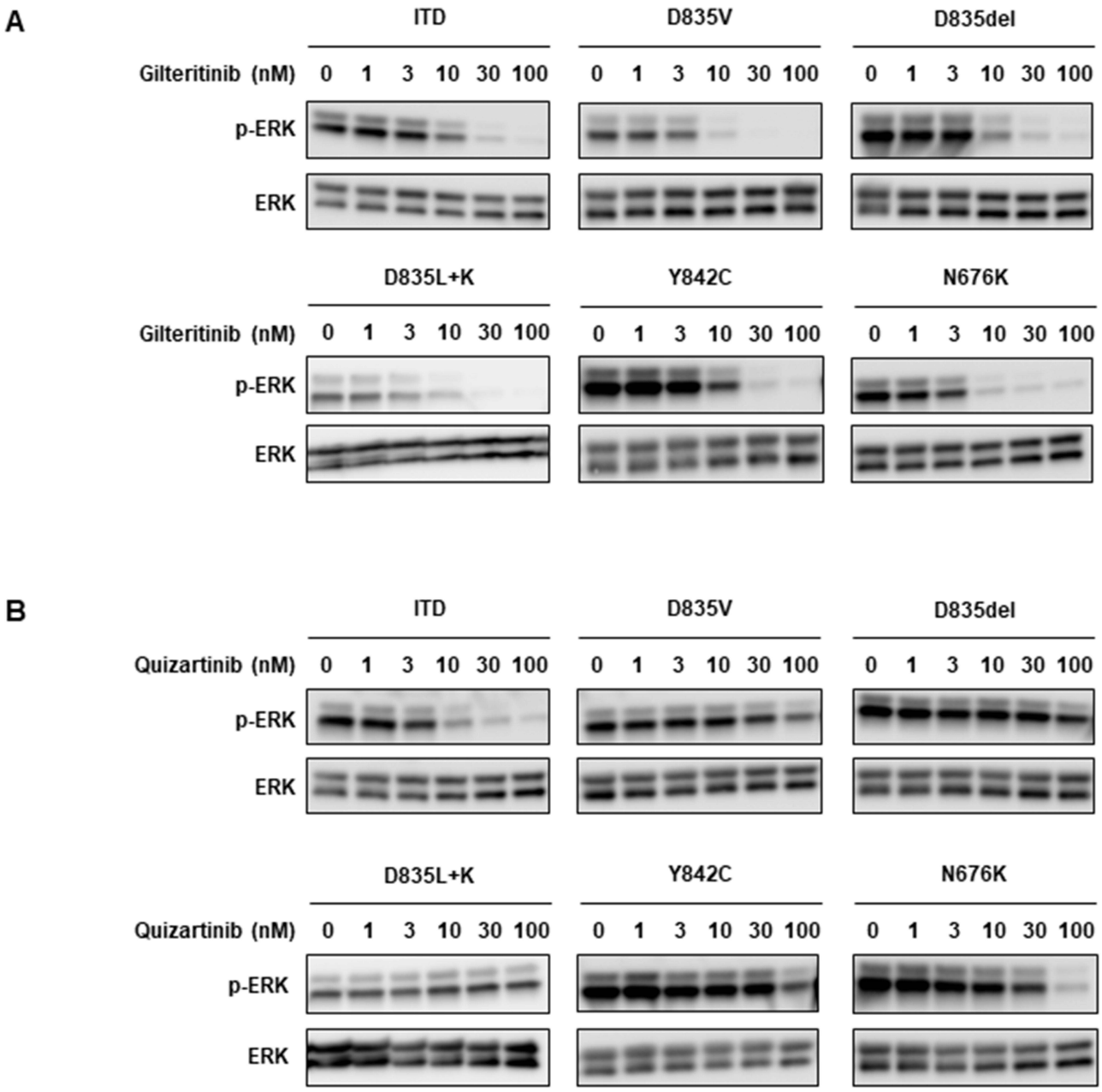

Figure 4 Inhibitory effect of gilteritinib (**A**) and quizartinib (**B**) on phosphorylated or total ERK in Ba/F3 cells expressing *FLT3*-ITD, *FLT3*-D835V, *FLT3*-D835del, D835L+K, Y842C or N676K.
Note: Mutant type FLT3-expressing Ba/F3 cells were treated with gilteritinib or quizartinib for 2 hours.
Abbreviations: ERK, extracellular signal-regulated kinase; FLT3, Fms-like tyrosine kinase 3; ITD, internal tandem duplication; p, phosphorylated.

antitumor effect of quizartinib was significantly attenuated in these mutant xenograft models (Figure 5). Once-daily oral administration of 30 mg/kg gilteritinib or 10 mg/kg quizartinib showed no effect on body weight in any of the mouse xenograft models (Supplementary Figure 1).

Discussion

In the present study, we demonstrate that gilteritinib exhibits potent in vitro and in vivo activity against a wide range of *FLT3*-TKD-PMs, including mutations known to confer resistance to midostaurin (namely N676K) or quizartinib (such as D835V and D835del).[2,19] Gilteritinib also showed in vitro activity against *FLT3*-JMD-PM comparable to its effect against *FLT3*-ITD mutations. In contrast, the inhibitory effect of quizartinib in the presence of D835V, D835L+K,

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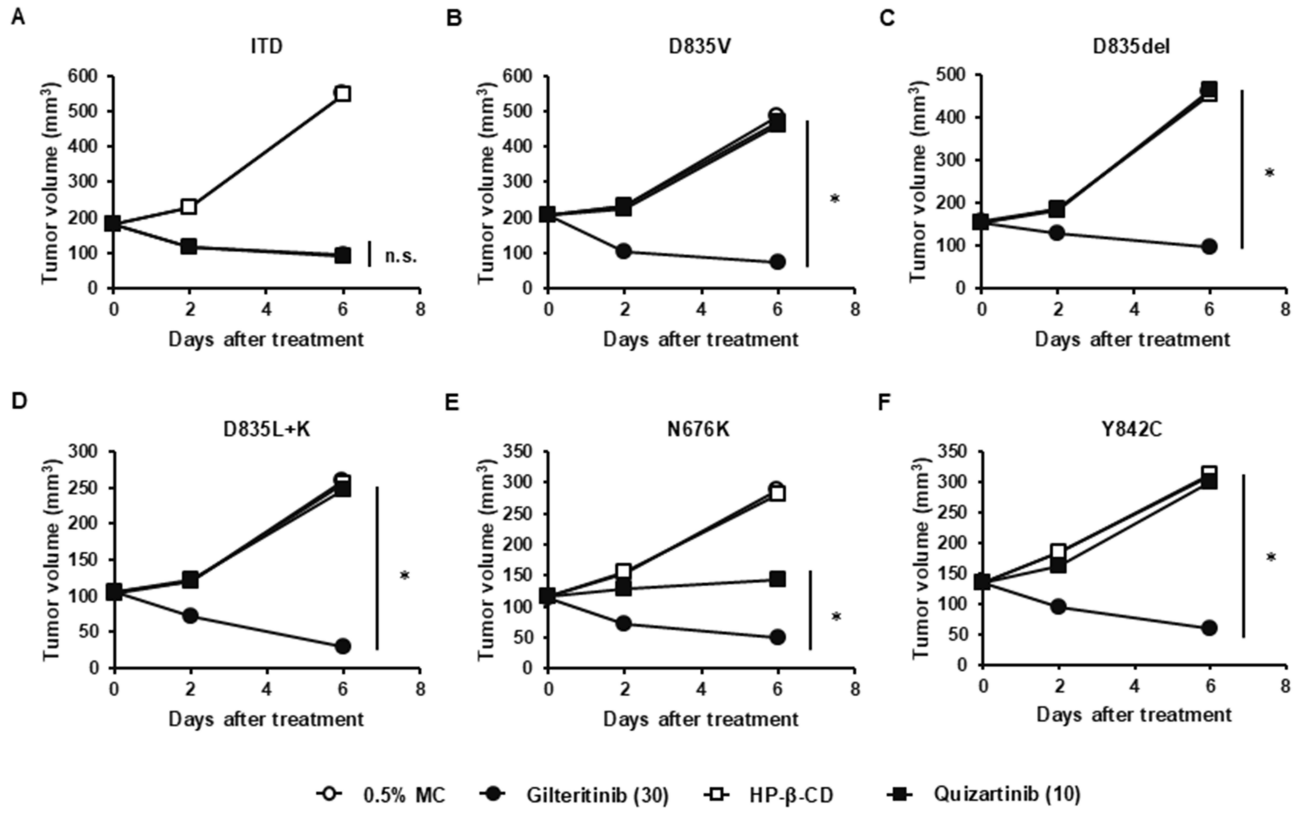

Figure 5 Antitumor activity of gilteritinib and quizartinib in a mutant FLT3-expressing Ba/F3-xenografted mouse model.Mice inoculated with Ba/F3 cells harboring mutant type FLT3 were orally administered gilteritinib at 30 mg/kg or quizartinib at 10 mg/kg, respectively (**A**) *FLT3*-ITD, (**B**) *FLT3* D835V, (**C**) *FLT3* D835del, (**D**) *FLT3* D835L+K, (**E**) *FLT3* N676K, (**F**) *FLT3* Y842C. Tumor volume was measured, and data are shown as means ± SEM (n = 4–5/group). Tumor volume on day 6 was compared between the gilteritinib-treated group and quizartinib-treated group using Student's *t*-test.

Note: *P < 0.05.

Abbreviations: FLT3, Fms-like tyrosine kinase 3; HP-β-CD, hydroxypropyl-β-cyclodextrin; ITD, internal tandem duplication; MC, methyl cellulose; SEM, standard error of the mean; n.s., not significant.

D835del, N676K or Y842C mutations was reduced by more than 6-fold compared with cells expressing *FLT3*-ITD mutations. The inhibitory effect of midostaurin in cells expressing N676K was also reduced by approximately 3-fold compared with its effect on cells expressing *FLT3*-ITD mutations.

Our findings align with previous research exploring the in vitro activity of gilteritinib.[3,17,18] An in vitro study that explored gilteritinib inhibitory activity across a broad spectrum of *FLT3* mutations showed that gilteritinib had potent inhibitory activity against both canonical and non-canonical *FLT3* mutations and limited vulnerability to resistance-causing *FLT3*-TKD-PMs, such as D835V, D835Y, Y842C and N676K.[3] A further study assessed the inhibitory activity of gilteritinib using a panel of *FLT3* point mutations known to confer resistance to type II inhibitors, such as sorafenib and quizartinib.[17] Here, gilteritinib demonstrated similar inhibitory activity against *FLT3*-TKD-PMs including D835H/Y/V, compared with cells expressing *FLT3*-ITD mutations.[17] Furthermore, another study showed that mutations at F691, D835 and Y842 were associated with resistance to quizartinib in samples obtained from patients with *FLT3*-ITD-mutated AML.[23] Additionally, we have elucidated the in vitro effects of gilteritinib on further *FLT3* mutations, including *FLT3*-JMD-PM, which have not been extensively studied pharmacologically. This novel focus broadens the scope of our research and enhances our understanding of AML, potentially positioning gilteritinib as a frontline therapy. Further investigation into the downstream signaling pathway changes and in vivo evaluations of these less-studied *FLT3* mutations will be essential to fully understand their clinical relevance and potential therapeutic implications.

In our study, gilteritinib also inhibited the autophosphorylation of FLT3 and its downstream molecules STAT5, AKT and ERK1/2 in Ba/F3 cells with *FLT3*-TKD-PMs D835V, D835del, D835L+K, Y842C and N676K, which have previously been shown to be resistant to quizartinib.[19] Again, these results are consistent with previous research,

where gilteritinib was associated with decreases in the phosphorylation levels of FLT3, STAT5, AKT and ERK in MV4–11 cells.[18]

The difference in selectivity between gilteritinib and quizartinib can be explained by variations in binding at the structural level.[12] Both gilteritinib and midostaurin are classified as type I FLT3 inhibitors that bind to the active conformation of the FLT3 receptor targeting either the activation loop or the ATP-binding pocket, and exhibit activity against both ITD mutations and TKD-PMs.[12] Type II FLT3 inhibitors, including quizartinib, bind to the FLT3 receptor in its inactive conformation near the ATP-binding domain; therefore, they do not target TKD-PMs.[12] While this study reinforces the efficacy of gilteritinib against *FLT3*-ITD mutations and *FLT3*-TKD-PMs, further mechanistic exploration is warranted to fully elucidate the reasons behind its superior efficacy compared to quizartinib. Structural analyses, including binding affinity studies and molecular docking simulation with resistant *FLT3* mutants, could provide deeper insights into these mechanisms.

Recent studies have also highlighted the role of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated oxidative stress signaling in the development of resistance to FLT3 inhibitors.[24] *FLT3*-ITD can trigger oxidative stress signaling pathways, including STAT5, PI3K/AKT, and RAS/MAPK, which promote cell survival and proliferation by regulating apoptosis-related genes and generating reactive oxygen species through NADPH oxidase.[24] This oxidative stress can lead to DNA damage and genomic instability, further contributing to drug resistance.[24] Targeting these oxidative stress pathways may offer new therapeutic strategies to overcome resistance in *FLT3*-ITD-mutated AML.[24] Although most research has focused on *FLT3*-ITD, similar resistance mechanisms may also affect other mutations, including TKD-PM.[24]

To further investigate the antitumor activity of gilteritinib against FLT3 inhibitor-resistant *FLT3*-TKD-PMs, we compared its efficacy to that of quizartinib using mutant xenograft mouse models. Both treatments effectively inhibited tumor growth in an *FLT3*-ITD xenograft model, without any body weight loss at the tested dose levels. However, the effect of quizartinib was significantly diminished in all examined *FLT3*-TKD-PM xenograft models, whereas gilteritinib maintained potent antitumor effects even in these models. This potent in vivo activity of gilteritinib against these resistant-conferring mutations highlights its potential as a more reliable treatment option for patients with FLT3 inhibitor-resistant mutations, particularly *FLT3*-TKD-PMs. Future clinical trials should evaluate the efficacy of gilteritinib both as monotherapy and in combination with other therapeutic agents to address multifaceted resistance mechanisms and improve patient outcomes. These efforts could lead to the development of comprehensive treatment strategies for AML, improving therapeutic options for patients with AML harboring resistant mutations.

In conclusion, this study demonstrates that gilteritinib is effective in vitro and in vivo against *FLT3*-TKD-PMs resistant to quizartinib or midostaurin, as well as a wide range of *FLT3* mutations, including TKD-PM and JMD-PM. These findings highlight the significance of the binding mechanism of gilteritinib as a type I inhibitor, suggesting its potential to overcome resistance to type II inhibitors like quizartinib. This underscores the need for further structural studies to explore the underlying mechanism of these differences in greater detail. Moreover, our comprehensive analysis of gilteritinib efficacy across multiple *FLT3* mutations provides valuable insights for the treatment of AML. These results indicate that gilteritinib could be integrated into treatment regimens for AML patients with resistant *FLT3* mutations or may serve as a frontline therapy, although the clinical relevance of these findings has yet to be fully determined. Further studies are warranted to assess the prognostic value of *FLT3*-TKD-PM and *FLT3*-JMD-PM, alongside the efficacy of gilteritinib in patients harboring these *FLT3*-TKD-PMs. Additionally, exploring effective combination strategies to overcome resistance will be crucial.

Abbreviations

AML, acute myeloid leukemia; ATP, adenosine triphosphate; BCA, bicinchoninic acid; CI, confidence interval; CO_2, carbon dioxide; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FL, FLT3 ligand; FLT3, Fms-like tyrosine kinase 3; HP-β-CD, hydroxypropyl-β-cyclodextrin; IC_{50}, half-maximal inhibitory concentration; IL-3, interleukin 3; ITD, internal tandem duplication; JMD, juxtamembrane domain; MAPK, mitogen-activated protein kinase; MC, methyl cellulose; NADPH, nicotinamide adenine dinucleotide phosphate; P, phosphorylated; PBS, phosphate buffered saline; PM,

point mutation; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM, standard error of the mean; STAT, signal transducer and activator of transcription; TKD, tyrosine kinase domain.

Data Sharing Statement

Researchers may request access to anonymized participant-level data, trial-level data and protocols from Astellas-sponsored clinical trials at www.clinicalstudydatarequest.com. For the Astellas criteria on data sharing see: https://clinicalstudydatarequest.com/Study-Sponsors/Study-Sponsors-Astellas.aspx.

Ethical Statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc., Tsukuba Research Center, which is accredited by AAALAC International.

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Author Contributions

All authors made a significant contribution to the work reported, whether that was in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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Disclosure

TN, TK, and MM are employees of Astellas. HT and TY were employees of Astellas at the time of this study, but are currently employees of Universal Cells, a subsidiary of Astellas Pharma Inc. The authors report no other conflicts of interest in this work.

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