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Gilteritinib Reduces FLT3 Expression in Acute Myeloid Leukemia Cells

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Abstract

Acute myeloid leukemia (AML) is a genetically diverse and challenging malignancy, with mutations in the FLT3 gene being particularly common and deleterious. Gilteritinib, a potent FLT3 inhibitor, has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of relapsed/refractory AML with FLT3 mutations. Although gilteritinib was developed based on its inhibitory activity against FLT3 kinase, it is important to understand the precise mechanisms of its antileukemic activity in managing drug resistance and discovering biomarkers. This study was designed to elucidate the effect of gilteritinib on the FLT3 expression level. The results showed that gilteritinib induced a dose-dependent decrease in both FLT3 phosphorylation and expression. This reduction was particularly pronounced after 48 h of treatment. The decrease in FLT3 expression was found to be independent of changes in FLT3 mRNA transcription, suggesting post-transcriptional regulatory mechanisms. Further studies were performed in various AML cell lines and cells with both FLT3 wild-type and FLT3 mutant exhibited FLT3 reduction by gilteritinib treatment. In addition, other FLT3 inhibitors were evaluated for their ability to reduce FLT3 expression. Other FLT3 inhibitors, midostaurin, crenolanib, and quizartinib, also reduced FLT3 expression, consistent with the effect of gilteritinib. These findings hold great promise for optimizing gilteritinib treatment in AML patients. However, it is important to recognize that further research is warranted to gain a full understanding of these mechanisms and their clinical implications in the context of FLT3 reduction.

Key Words: Gilteritinib, FLT3, Acute myeloid leukemia, Antileukemic activity, FLT3 expression, Kinase inhibitors

INTRODUCTION

Acute myeloid leukemia (AML) is a hematopoietic malignancy identified by the clonal expansion of immature myeloid progenitor cells in bone marrow, blood, and other tissues. It is a highly heterogeneous disease with various genetic abnormalities, and prognosis varies significantly depending on the patient's age, general health, and specific cytogenetic and molecular features (DiNardo and Cortes, 2016; Pollyea *et al*., 2023). Mutations in the FLT3 gene are among the most frequent and essential genetic abnormalities linked to AML (Meshinchi and Appelbaum, 2009; Levis, 2013; Daver *et al*., 2019).

FLT3 is a receptor tyrosine kinase that performs a crucial function in preserving hematopoietic stem cell equilibrium while also regulating the proliferation and viability of hematopoietic progenitor cells (Cao *et al*., 2021). FLT3 mutations are prevalent in about one-third of AML patients, and the most frequent mutation is FLT3 internal tandem duplication (FLT3-ITD). The constitutive activation of FLT3 signaling and promotion of leukemogenesis results from an in-frame insertion of tandem repeats of the juxtamembrane domain of the FLT3 receptor known as FLT3-ITD. FLT3-ITD has been associated with poor prognosis, heightened risk of relapse, and lower survival rates (Daver *et al*., 2019; Tao *et al*., 2019).

Gilteritinib is a potent and selective FLT3 inhibitor that has been approved by the U.S. Food and Drug Administration for the treatment of relapsed/refractory AML with FLT3 mutations. The drug inhibits FLT3 autophosphorylation and downstream signaling pathways, causing cell cycle arrest, apoptosis, and inhibition of cell proliferation. Compared to other FLT3 inhibitors, including sorafenib, midostaurin, and quizartinib, gilteritinib offers superior potency, selectivity, and tolerazbility (Gunawardane *et al*., 2013; Mori *et al*., 2017; Weisberg *et al*., 2019; Levis and Perl, 2020).

Although gilteritinib was developed based on its inhibitory

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activity against FLT3 kinase, it is imperative to elucidate the underlying mechanisms of its antileukemic activity. Understanding the precise mechanisms that contribute towards its antileukemic activity is crucial in managing drug resistance and discovering biomarkers. Hence, this study aims to investigate the effect of gilteritinib on FLT3 expression in AML cells as a novel mechanism.

MATERIALS AND METHODS

Cell lines and reagents

Three AML cell lines MOLM-14, EOL-1, and MOLM-13 were obtained from DSMZ (Braunschweig, Germany) and HL-60 cells were purchased from Korean cell line bank (Seoul, Korea). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO2 at 37°C.

Gilteritinib (HY-12432), midostaurin (HY-10230), crenolanib (HY-13223), and quizartinib (HY-13001) were purchased from MedChemExpress (Monmouth Junction, NJ, USA).

Western blot analysis

Cells were harvested and lysed in RIPA buffer (Biosessang, Yongin, Korea) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, MA, USA). Protein concentration was determined using the bicinchoninic acid assay kit (SMART™ BCA Protein Assay Kit, iNtRON Biotechnology, Seongnam, Korea). Cell lysates were separated using SDS-PAGE and transferred to Immobilon®-P polyvinylidene difluoride (PVDF) membranes (0.45 μm, Millipore, Darmstadt, Germany). The membranes were then blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 buffer and probed with primary antibodies against FLT3, pFLT3 (Cell sinaling Technology, MA, USA), and β-actin (Santa Cruz Biotechnology, TX, USA).

After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized using an enhanced chemiluminescence (Amersham, GE Healthcare, NJ, USA) substrate.

qRT-PCR

Total RNA was extracted from MOLM-14 cells using ReliaPrep™ RNA Miniprep Systems (Promega, WI, USA), according to the manufacturer's instructions. Subsequently, cDNA was synthesized using the SuperScript IV First-Strand Synthesis System, and qRT-PCR was performed using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, MA, USA) to measure FLT3 mRNA expression levels using the primers (5'-TGGACCTTCTCTCGAAAATCATTT-3', and 5'-GCATCATCATTTTCTGCATGGA-3').

RESULTS

The effects of gilteritinib on FLT3 phosphorylation and expression in MOLM-14 AML cell lines were evaluated. MOLM-14 cells harbor the FLT3-ITD mutation and express a high level of FLT3 protein (Quentmeier *et al*., 2003). The cells were treated with different amounts of gilteritinib and analyzed using western blot. As expected, gilteritinib reduced the phosphorylated form of FLT3 dose-dependently (Fig. 1A). Moreover, the expression level of FLT3 was also reduced by gilteritinib treatment in a dose-dependent manner. Time-course experiments with gilteritinib treatment were performed. As shown in Fig. 1B, FLT3 phosphorylation was inhibited as early as 4 h after treatment. Along with p-FLT3 level reduction, FLT3 expression level was decreased by gilteritinib with a maximal effect at 48 h of treatment.

To figure out if FLT3 expression level is reduced at transcription, FLT3 mRNA level was measured. qRT-PCR experiment performed after the gilteritinib treatment indicates that gilteritinib did not reduce transcription of FLT3 mRNA (Fig. 2). Gilteritinib rather slightly induced FLT3 transcription, exhibiting that the change in FLT3 protein expression level is not caused by transcriptional change.

FLT3 expression decrease by gilteritinib was also observed in other AML cell lines, including MOLM-13, EOL-1 and HL-60 cells (Fig. 3). MOLM-13 cells express FLT3-ITD mutant, while EOL-1 and HL-60 cells have wild-type of FLT3 protein (Quentmeier *et al*., 2003; Ampasavate *et al*., 2019). As shown in Fig. 3A, gilteritinib reduced FLT3 expression at a concentration as low as 10 nM in MOLM-13 cells. Additionaly, FLT3 phosphorylation is also inhibited by gilteritinib in MOLM-13 cells. In EOL-1 and HL-60 cells, gilteritinib also suppressed FLT3 expres-

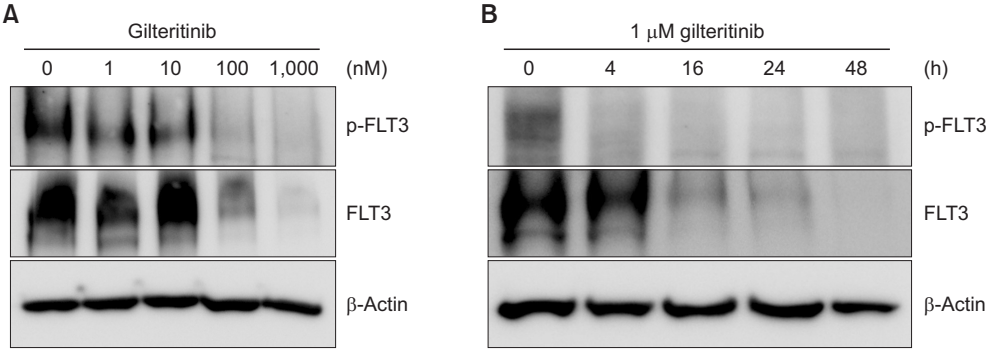

Fig. 1. Gilteritinib reduces FLT3 expression. (A) Western blot analysis of FLT3 and p-FLT3 expression in MOLM-14 cells treated with different concentrations of gilteritinib for 24 h. β-Actin was used as a loading control. (B) Western blot analysis of FLT3 and p-FLT3 expression in MOLM-14 cells treated with 1 μM gilteritinib for the indicated time. β-Actin was used as a loading control.

sion, but only at a higher concentration of gilteritinib (Fig. 3B, 3C). In MOLM-13 and MOLM-14 cells, the FLT3 western blot band disappeared almost completely with 1 μM of gilteritinib, whereas a concentration of 10 μM of gilteritinib was needed to down-regulate FLT3 in EOL-1 and HL-60 cells. Notably, EOL-1 and HL-60 cells lacked FLT3 activating mutation as p-FLT3 bands were undetected.

The effects of other FLT3 inhibitor on the FLT3 protein level were determined. Midostaurin, crenolanib, and gilteritinib are type I FLT3 inhibitors that bind to the active conformation of FLT3 (Acharya *et al*., 2022; Zhao *et al*., 2022). Conversely, quizartinib is a type II FLT3 inhibitor that binds to the inactive conformation of FLT3 (Stone *et al*., 2017; Zhang *et al*., 2019; Zhao *et al*., 2019). All four FLT3 inhibitors tested showed the reduction in FLT3 expression (Fig. 4). Among the FLT3 inhibitors, quizartinib exhibited a relatively weaker effect. These results indicate that gilteritinib has demonstrated the ability to decrease FLT3 expression in AML cells, which may contribute to its antileukemic properties.

DISCUSSION

Our study provides new insight into the underlying mechanism of gilteritinib in AML treatment, demonstrating that gilteritinib reduces FLT3 expression in AML cells, which might contribute to its antileukemic efficacy (Levis and Perl, 2020). Our findings are consistent with previous studies suggesting that reducing FLT3 expression is a novel mechanism of action for drugs targeting FLT3 and might be a more effective strategy than simply inhibiting FLT3 signaling (Cao *et al*., 2021; Chen *et al*., 2022)

Reduced FLT3 protein level by gilteritinib treatment may be caused by FLT3 degradation, and it is highly probable. However, demonstrating FLT3 degradation is not amenable due to experimental limitations. To exhibit proteasomal degradation of FLT3, treatment with the proteasome inhibitor bortezomib is required. However, treatment with bortezomib itself induces FLT3 degradation via the autophagy mechanism (Larrue *et al*., 2016). MG132, another proteasome inhibitor, also downregulated FLT3 protein expression (unpublished data). Because of these effects of proteasome inhibitors on FLT3 degradation, it could not be determined whether or not gilteritinib degrades FLT3 protein.

The effects of gilteritinib on FLT3 expression appear to be related to the status of FLT3 phosphorylation. Given that a higher concentration of gilteritinib is required for FLT3 downregulation in EOL-1 and HL-60 cells than in MOLM-13 and MOLM-14 cells (Fig. 1, 3), FLT3-ITD appears to be more susceptible than FLT3-wt to gilteritinib. Previous papers regarding FLT3 degradation has also reported the susceptibility of FLT3-ITD compared to FLT3-wt to small molecule degraders (Minami *et al*., 2002; Ly *et al*., 2013; Wang *et al*., 2017; Katayama *et al*., 2018). Further meticulous experiment design will be required to determine susceptibility to FLT3 mutants and wild-types.

Other known FLT3 inhibitors were tested for their effect on FLT3 expression, including midostaurin, crenolanib, and quizartinib. Midostaurin, a potent kinase inhibitor, is a key

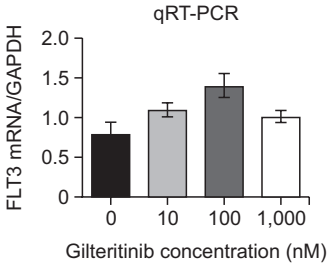

Fig. 2. qRT-PCR analysis of FLT3 mRNA expression levels in MOLM-14 cells. Cells were treated with different concentrations of gilteritinib for 24 h. Results are expressed as mean ± SEM of three independent experiments.

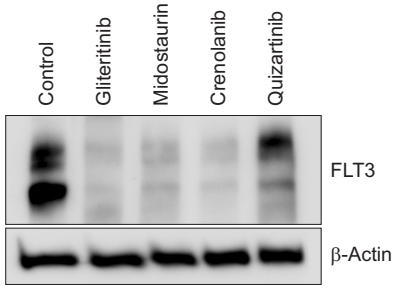

Fig. 4. Other FLT3 inhibitors also reduce FLT3 expression. Western blot analysis of FLT3 expression in MOLM-14 cells treated with 1 μM of gilteritinib, midostaurin, crenolanib, or quizartinib for 24 h. β-Actin was used as a loading control.

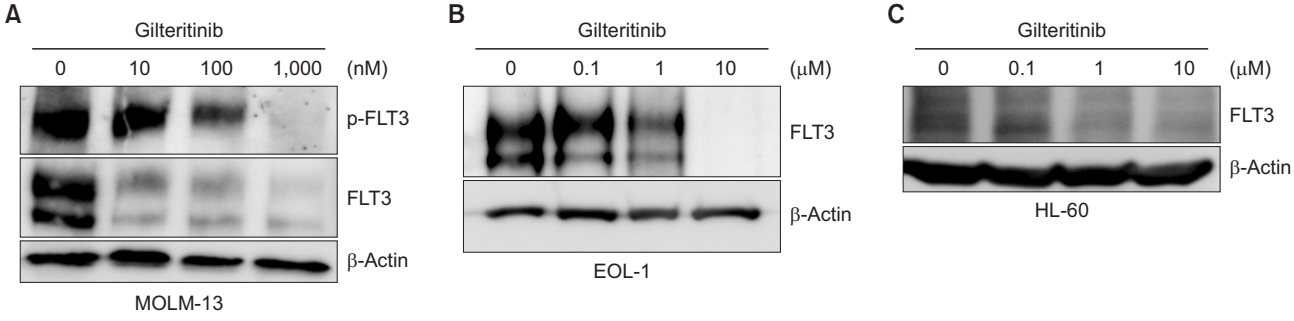

Fig. 3. Gilteritinib reduces FLT3 expression in other AML cell lines. Western blot analysis of FLT3 expression in (A) MOLM-13, (B) EOL-1, and (C) HL-60 cells treated with different concentrations of gilteritinib for 24 h. β-Actin was used as a loading control.

player in the treatment of AML. Midostaurin's approved status allows it to be used effectively in the treatment of AML patients with FLT3 mutations, whether newly diagnosed, relapsed or refractory (Stone *et al*., 2017). Crenolanib is a highly selective tyrosine kinase inhibitor that primarily targets FLT3 and shows exceptional efficacy against the challenging FLT3-ITD mutations. (Galanis *et al*., 2014). Quizartinib, a highly selective FLT3 inhibitor, targets FLT3-ITD mutations by inhibiting the FLT3 kinase's inactive conformation as a type II inhibitor (Cortes *et al*., 2018). At 1 μM concentration, midostaurin and crenolanib reduced FLT3 expression to a similar extent as gilteritinib, while quizartinib showed a weak effect on the FLT3 expression compared to gilteritinib. The binding affinities (Kd values) of these inhibitors to FLT3 protein are reported: 1.0 nM (giltertitinb), 7.9 nM (midostaruin), 0.28 nM (crenolarnib), and 1.1 nM (quizartinib) (Levis and Perl, 2020). Kd values for gilteritinib and quizartinib are similar value, suggesting that binding affinity are not related to FLT3 downregulating effect. Quizartinib is type II FLT3 inhibitor and gilteritinib, midostaurin, and crenolanib are type I inhibitors (Shimada, 2019). This difference in binding mode may be the reason for the difference in activity for FLT3 reduction. However, further studies are needed to understand the mechanism of FLT3 reduction and the differences in activity between FLT3 inhibitors.

This study reports the novel mechanism of gilteritinib, FLT3 protein reduction. Given that target protein degraders such as PROTAC are generally considered to be more effective than enzyme activity inhibitors (Ohoka *et al*., 2022), this novel property of gilteritinib will be added value for antileukemic effects. At this point, it is not clear whether FLT3 reduction is beneficial in terms of adverse events when used in AML patients. Both FLT3 mutant and wild type proteins appear to be affected by gilteritinib (Fig. 3). This suggests that cells with FLT3 wild type without activating mutation may be disturbed by gilteritinib, which may be a cause of adverse events.

There are several clinical implications from the present study in gilteritinib treatment, given the increasing use of gilteritinib to AML patients (Cortes *et al*., 2018; Levis and Perl, 2020; Katagiri *et al*., 2023; Martinez-Gutierrez *et al*., 2023). First, the expression level of FLT3 protein as well as the presence of FLT3 mutation can be marker for gilteritinib response. There may be a correlation between gilteritinib response and FLT3 expression level in leukemic cells potentially. Second, tissues with high FLT3 expression (lymph node, spleen, bone marrow) may be affected by gilteritinib as well as cells with FLT3 mutation, which may result in adverse effects in gilteritinib-treated patients. Those will be subjects of clinical research required for optimal treatment of gilteritinib for better clinical outcome.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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