Gilteritinib-induced upregulation of S100A9 is mediated through BCL6 in acute myeloid leukemia

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Drug resistance and relapse are common challenges in acute myeloid leukemia (AML), particularly in an aggressive subset bearing internal tandem duplications (ITDs) of the FLT3 receptor (FLT3-ITD). The tyrosine kinase inhibitor gilteritinib is approved for the treatment of relapse/refractory AML with FLT3 mutations, yet resistance to gilteritinib remains a clinical concern, and the underlying mechanisms remain incompletely understood. Using transcriptomic analyses and functional validation studies, we identified the calcium-binding proteins S100A8 and S100A9 (S100A8/A9) as contributors to gilteritinib resistance in FLT3-ITD AML. Exposure of FLT3-ITD AML cells to gilteritinib increased S100A8/A9 expression in vivo and in vitro and decreased free calcium levels, and genetic manipulation of S100A9 was associated with altered sensitivity to gilteritinib. Using a transcription factor screen, we identified the transcriptional corepressor BCL6, as a regulator of S100A9 expression and found that gilteritinib decreased BCL6 binding to the S100A9 promoter, thereby increasing S100A9 expression. Furthermore, pharmacological inhibition of BCL6 accelerated the growth rate of gilteritinib-resistant FLT3-ITD AML cells, suggesting that S100A9 is a functional target of BCL6. These findings shed light on mechanisms of resistance to gilteritinib through regulation of a target that can be therapeutically exploited to enhance the antileukemic effects of gilteritinib.

Introduction

An aggressive subset of acute myeloid leukemia (AML)—bearing internal tandem duplications (ITDs) of the FLT3 receptor (FLT3-ITD) occurs in ~30% of adult patients and is associated with poor prognosis and low overall survival. Gilteritinib, a tyrosine kinase inhibitor of FLT3, AXL, and ALK, has been approved for the treatment of FLT3-mutated AML, yet drug resistance remains a clinical concern. We sought to understand gilteritinib-resistance mechanisms in FLT3-ITD AML, and identified the calcium-binding protein S100A9 as a potential therapeutic target mediating resistance.

S100A8 and S100A9 are constitutively expressed in myeloid cells and have roles in cell differentiation, autophagy, and apoptosis. Overexpression of S100A8 in AML is associated with poor survival, and altered expression of S100A8 and/or S100A9 confers resistance or reduced sensitivity to agents in AML therapy, including venetoclax, doxorubicin, vincristine, and etoposide, but they have yet to be studied in the context of gilteritinib resistance. Although changes in autophagy and apoptosis may play a

Key Points

- S100A9 overexpression promotes gilteritinib resistance in FLT3-ITD AML cells.
- Gilteritinib-induced upregulation of S100A9 is mediated through loss of BCL6 enrichment at the S100A9 promoter.
role in drug response, the exact mechanisms of S100A8/A9 in drug resistance remain poorly understood. Furthermore, the transcription factors that govern S100A8/A9 expression in AML remain unknown. We identified a connection of gilteritinib-induced S100A9 expression with BCL6, a transcriptional corepressor, to promote drug resistance in FLT3-ITD+ AML.
Methods

Xenograft mouse models were generated and treated as previously described.16 Human FLT3-ITD+ AML cell lines (MOLM13 and MOLM13-RES), a murine cell line (BAF3) containing human FLT3-ITD or FLT3-ITD/D835Y, and human primary AML samples were treated with gilteritinib, PLX51107, tasquinimod, and BI-3802, separately or in combination, before sample collection or an assay was performed. Details are in the supplemental Methods. All animal studies were reviewed and approved by The Ohio State University (OSU) Institutional Animal Care and Use Committee. Deidentified, genomically annotated human primary AML samples were obtained after written informed consent was received under an OSU Institutional Review Board–approved protocol and in accordance with the Declaration of Helsinki.

Results and discussion

Gilteritinib induces expression of S100A8/A9 in vitro and in vivo

We used RNA sequencing (RNA-seq) analysis to identify alternative mechanisms of gilteritinib resistance. Two FLT3-ITD+ AML cell lines (MOLM13 and MOLM13-RES, a resistant cell line bearing the
**FLT3 D835Y mutation** were xenografted into NSG mice and treated with gilteritinib until leukemia progression.16,18 Leukemic cells were isolated from the bone marrow, and gene expression analyses indicated that S100A8 and S100A9 (S100A8/A9) were 2 of the top 25 genes upregulated after gilteritinib treatment in both models (S100A9 ranked 11th and 5th, and S100A8 ranked 14th and 20th in MOLM13 and MOLM13-RES xenografts, respectively; Figure 1A). We confirmed this transcriptional upregulation by reverse transcription-polymerase chain reaction (RT-PCR; Figure 1B), but only S100A9 protein was significantly increased in the MOLM13-RES model (Figure 1C; supplemental Figure 1A).

Because S100A8/A9 were significantly increased after drug treatment in vivo, we interrogated their response during gilteritinib treatment in vitro in 4 FLT3-ITD+ cell lines: MOLM13, MOLM13-RES, and murine BAF3 cells expressing human FLT3-ITD or FLT3-ITD with a D835Y mutation (FLT3-ITD/D835Y). S100A8 and S100A9 transcripts were significantly upregulated in all cell lines after acute gilteritinib exposure (Figure 1D). Furthermore, ex vivo gilteritinib treatment of a human primary AML sample with FLT3-ITD significantly increased both transcripts (supplemental Figure 1B), and serial primary AML samples collected from 2 patients treated with gilteritinib had a significant increase in S100A9 (supplemental Figure 1C). Interestingly, S100A9 protein expression was increased after gilteritinib treatment in MOLM13 and MOLM13-RES cells in a time-dependent manner, whereas S100A8 had minimal change (Figure 1E). Because these small, soluble proteins can form homodimers, heterodimers, and oligomers that bind and regulate calcium,17,18 we used an intracellular free-calcium assay to assess the calcium levels after gilteritinib treatment. Our results indicate that free calcium significantly decreased after acute exposure to gilteritinib (Figure 1F), presumptively because of sequestration by S100A8/A9. This observation is consistent with reports of decreased calcium levels or release after venetoclax (a BCL-2 inhibitor) treatment in AML12 or in prednisolone-resistant MLL-rearranged infant ALL when S100A8/A9 are overexpressed.19

**Modulation of S100A9 alters sensitivity to gilteritinib in vitro and ex vivo**

Because S100A9 expression was more consistently and significantly changed than S100A8 in our models, we focused our subsequent efforts on determining the functional significance of gilteritinib-induced S100A9 expression in FLT3-ITD+ AML. We assessed cell growth in the presence of gilteritinib after overexpression or knockdown of S100A9 in MOLM13 cells (Figure 1G-H; supplemental Figure 1D-G). Cells with overexpression of S100A9 were less sensitive to gilteritinib than those expressing the control vector (Figure 1G). Conversely, cells after knockdown of S100A9 were more sensitive to gilteritinib than were cells with empty vector or the non-targeting control (Figure 1H). In addition, pharmacological targeting of S100A9 with tasquinimod, a quinolone-3-carboxamide that binds and inhibits S100A9,10,20 partially sensitized MOLM13 cells to gilteritinib (supplemental Figure 1H). More recently, BET inhibition has been shown to suppress S100A8/A9,10,21 and in line with this observation, we found that cotreatment of the BET inhibitor PLX51107 with gilteritinib suppressed growth of MOLM13 cells when compared with either drug alone (Figure 1I; supplemental Figure 1I). Furthermore, PLX51107 sensitized human primary AML cells to gilteritinib, whereas tasquinimod did not enhance the antileukemic effect of gilteritinib in the patient samples (Figure 1J; supplemental Figure 1J). Collectively, the data indicate that overexpression of S100A9 confers resistance to gilteritinib. It should be noted that S100A8 has been found to enhance resistance to etoposide and vincristine by modulating apoptosis and autophagy pathways13,14 and can drive proliferation, whereas S100A9 promotes differentiation through TLR4 in AML.22 Our present results add to this prior knowledge and indicate an effect of S100A9 on cell growth that promotes a gilteritinib-resistance phenotype that can be targeted by genetic or pharmacological inhibition.

**Gilteritinib-induced upregulation of S100A9 is mediated through loss of BCL6 enrichment at the S100A9 promoter**

To identify the transcriptional regulators that govern gilteritinib-induced upregulation of S100A8/A9, we performed a transcription factor (TF) screen by cotransfecting HEK293 cells with expression vectors of 1623 transcriptional regulators (supplemental Figure 2A) and S100A8/A9 promoter luciferase constructs. TFs that increased or decreased promoter activity by greater than twofold were considered potential candidates (Figure 2A). Narrowing our focus to top TFs with overlapping effects on both S100A8 and S100A9 promoter activity revealed RUNX2 and BCL6 as 2 candidate transcriptional regulators (Figure 2A). Further validation indicated that RUNX2 enrichment at the S100A9 promoter was unchanged after gilteritinib treatment (supplemental Figure 2B), whereas BCL6 overexpression resulted in a significant reduction in both S100A8/A9 promoter activity (Figure 2B), as well as significantly decreased BCL6 enrichment at the S100A8/A9 promoters in gilteritinib-treated MOLM13 cells (Figure 2C; supplemental Figure 2C). Furthermore, accessibility at the S100A9 promoter significantly increased after gilteritinib treatment in vivo (supplemental Figure 3A), whereas there was no significant change at the S100A8 promoter (supplemental Figure 3B). These results indicate that gilteritinib decreases localization of BCL6 to the S100A9 promoter.

The BCL6 inhibitor BI-3802 promotes degradation of BCL6.23 BI-3802–mediated BCL6 degradation was not cytotoxic in MOLM13 cells at a concentration of 1 μM (Figure 2D), but increased S100A8/A9 transcripts and S100A9 protein (Figure 2E-F). Because BCL6 binds and represses the transcriptional activation of S100A9, we used BI-3802 to emulate the S100A9-overexpression model. Pretreatment followed by cotreatment of BI-3802 with gilteritinib caused MOLM13 cells to grow at a significantly accelerated rate, compared with gilteritinib treatment alone (Figure 2G), which may have been due to enhanced upregulation of S100A9. Collectively, these data indicate that BCL6 negatively regulates S100A9 expression, but gilteritinib promotes the dissociation of BCL6 from the S100A9 promoter.

In summary, our data support a novel mechanism of gilteritinib-induced upregulation of S100A9 that is mediated through BCL6. Through a currently unknown mechanism, gilteritinib promotes BCL6 dissociation from the S100A9 promoter, allowing for upregulation of S100A9 that promotes cell growth and a resistance phenotype. Our studies provide insight into the transcriptional regulation
of S100A9 that can be further evaluated across a range of therapies and cancers and exploited for potential targeting in combinatorial treatment strategies to prevent or overcome drug resistance.

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