



Communication

An HSP90 Inhibitor Overcomes FLT3 Inhibitor Resistance in FLT3/ITD-Positive Leukemia Cells with an N676K Mutation

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Abstract: *FLT3* mutations are frequently identified in acute myeloid leukemia (AML). In particular, *FLT3*-ITD is known to be an indicator of a poor prognosis. *FLT3* inhibitors have improved the treatment outcomes of AML patients with mutated *FLT3*. However, several drug-resistance mechanisms have been reported, and new clinical strategies to overcome drug resistance are needed. Heat shock protein (HSP) 90 is a molecular chaperone that mediates the correct folding and functionality of its client proteins, including *FLT3*. In the present study, we investigated the effects of an HSP90 inhibitor on *FLT3* inhibitor-resistant AML cells. Using MOLM-13 (an AML cell line harboring *FLT3*-ITD), we established *FLT3*-selective inhibitor (FI-700)-resistant cell lines with an *FLT3* N676K mutation. An HSP90 inhibitor (17-AAG) inhibited the growth of the cell lines, and combination treatment with FI-700 and 17-AAG showed synergistic inhibition. The underlying mechanism is thought to be as follows: HSP90 inhibits the association between HSP90 and *FLT3*, and thus reduces the phosphorylation of *FLT3* and its downstream signaling proteins, which induces the consequent degradation of *FLT3*. In summary, we demonstrated that the HSP90 inhibitor could inhibit the cell growth of *FLT3* inhibitor-resistant AML cells. Our results suggest that HSP90 is a promising molecular target in relapsed/refractory AML.

Keywords: acute myeloid leukemia; *FLT3* mutation; heat shock protein 90



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1. Introduction

FMS-like tyrosine kinase 3 (*FLT3*) is classified as a type 3 receptor tyrosine kinase, along with KIT, FMS, and PDGFR [1–3]. *FLT3* ligands induce the phosphorylation of *FLT3* on the surface of hematopoietic stem cells and progenitor cells, inducing proliferation and inhibiting apoptosis. Several types of mutations have been identified in acute myeloid leukemia (AML), and internal tandem duplications (ITD) and mutations in the tyrosine kinase domain (TKD) are observed in approximately 20% and 10% of AML patients, respectively [4–6]. In particular, *FLT3*-ITD is known to be a major indicator of a poor prognosis [7]. Clinical trials have shown that multiple *FLT3*-selective inhibitors contribute to improving the prognosis of both newly diagnosed and relapsed/refractory AML with *FLT3* mutations [8–10]. However, several resistance mechanisms have become apparent, and further clinical strategies to overcome drug resistance are needed.

Heat shock protein 90 (HSP90) acts as a molecular chaperone that plays an important role in mediating the proper folding and functionality of its client proteins in cells. More than 400 proteins have been reported to be client proteins, and many of them are essential for constitutive cell signaling and adaptive responses to stress [11]. A large number of client proteins are involved in oncogenesis and the progression of cancer [12]. Furthermore, in various cancer cells, the HSP90 expression levels are much higher than in their normal counterparts [13]. Thus, HSP90 may be critically important for cancer growth and progression. HSP90 inhibitors have been developed and are undergoing clinical trials in various cancers. One mechanism of HSP90 inhibitors is the blocking of ATP binding, which leads to changes in the HSP90–client protein complex, and the degradation of target proteins [14,15].

FLT3 and its mutant protein have been reported to be client proteins of HSP90. We hypothesized that HSP90 may be an actionable target for FLT3 inhibitor-resistant AML. In the present study, we examined the effects of an HSP90 inhibitor in an FLT3 inhibitor-resistant leukemia cell line.

2. Materials and Methods

2.1. Cell Cultures and Establishment of FLT3 Inhibitor-Resistant Cell Lines

MOLM-13, a cell line of AML-M5a harboring FLT3-ITD, was kindly provided by Dr. Yoshinobu Matsuo (Fujisaki Cell Center, Okayama, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco-BRL, Gaithersburg, MD, USA). To establish FLT3 inhibitor-resistant cell lines, cells were cultured with gradually increasing doses of FI-700 (5-(5-(piperazin-1-ylmethyl)-1,3,4-oxadiazol-2-yl)-N4-propyl-N2-(2-(pyridin-4-yl)ethyl)pyrimidine-2,4-diamine, Kyowa Hakko Kogyo, Shizuoka, Japan) from 0.01 μ M to 1.0 μ M. After several weeks, heterogenous 1.0 μ M FI-700-resistant cells were seeded in a 96-well plate for limiting dilution and further cultured under FI-700 treatment. We selected six clones and maintained them in RPMI 1640 medium supplemented with 10% fetal calf serum and 1.0 μ M FI-700.

2.2. Sanger Sequencing and Allele-Specific PCR of FLT3

Total RNA was extracted from cells using the QIAamp RNA Blood Mini Kit (QIAGEN, Tokyo, Japan). cDNA was synthesized from each RNA sample using random primers and Molony murine leukemia virus reverse transcriptase (SUPERScript II; Invitrogen, Carlsbad, CA, USA).

2.3. Cell Growth Assay and Apoptosis Assay

The viability of cells was estimated using the MTT assay. The cells were seeded into 96-well plates, then 17-AAG (17-N-allylamino 17-dimethoxy-geldanamycin, also known as Tanespimycin) was added at various concentrations in triplicate, and the plates were then incubated for 72 h. Subsequently, MTT solution was added to each well. After incubation for 3 h, cell viability was assessed.

The apoptotic rates were analyzed using the sub-G1 assay and Annexin V assay after treatment with drugs for 48 h. For the DNA histogram analysis, cells were washed with phosphate-buffered saline (PBS) and re-suspended in PBS containing 0.2% Triton X-100 and 50 μ g/mL PI (Sigma Chemical, St. Louis, MO, USA). Subsequently, flow cytometry was conducted on a FACScan (Becton Dickinson, San Jose, CA, USA). To detect the early stage of apoptosis, an Annexin-V-FLUOS Staining Kit (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instructions.

2.4. Flow Cytometry Analysis of FLT3 Expression

Cells were treated with drugs for 6 h, then washed with FACS buffer, and resuspended in FACS buffer containing phycoerythrin-conjugated anti-human FLT3 monoclonal antibody (Immunotech, Marseille, France). After incubation for 30 min on ice, the cells were washed twice and analyzed using FACScan.

2.5. Immunoprecipitation and Immunoblot Analysis

Anti-phospho MAPK, anti-MAPK, anti-phospho Akt, and anti-Akt antibodies were obtained from New England Biolabs (Beverly, MA, USA). Anti-FLT3 and anti-STAT5a antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Anti-phospho-STAT5 antibody was purchased from Kyowa Hakko Kogyo. Murine anti-phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-actin antibody was purchased from Roche Diagnostics (Tokyo, Japan).

After treatment with FI-700 and/or 17-AAG for 6 h, cells were washed twice with ice-cold PBS and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 1 mM Na₃VO₄, and 1 mM phenylmethylsul-

fonyl fluoride). After incubation for 1 h at 4 °C, the lysates were spun at 12,000 g and 4 °C for 20 min, and the pellets were subsequently discarded. A portion of the lysed samples was immunoprecipitated with each antibody using protein G Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). The precipitated or non-precipitated samples were separated using SDS-polyacrylamide gel electrophoresis and electroblotted onto Immobilon PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked overnight at 4 °C with 1% blocking reagent (Boehringer Mannheim) in 0.1 M maleic acid and 0.15 M NaCl (pH 7.5). The membranes were then incubated with the antibodies for immunodetection for 1 h. HRP-labeled goat anti-mouse and rabbit IgG antibodies (Amersham Pharmacia Biotech) and ECL chemiluminescent reagents (Amersham Pharmacia Biotech) were used for the detection of the signals.

3. Results

3.1. Establishment of MOLM-13 Sublines with a Resistance Mutation to an FLT3-Selective Inhibitor

To generate FLT3 inhibitor-resistant AML cell lines, we cultured MOLM-13 cells (an FLT3-ITD-positive human acute monocytic leukemia cell line) with increasing doses of FI-700 (from 0.01 µM to 1 µM) for several weeks. FI-700 has been developed as a selective kinase inhibitor against FLT3 and has been shown to have antileukemia activity both in vivo and in vitro, dependent on the dephosphorylation of constitutively active mutant FLT3 kinases [16]. Using limiting dilution in the presence of 1 µM FI-700, six clones with resistance to more than 1 µM FI-700 were established from the cells with heterogeneous resistance. We sequenced the *FLT3* of these clones, and identified an N676K mutation that was newly acquired in one allele in all six clones (Figure 1A). Similarly, allele-specific polymerase chain reaction (PCR) of the resistant clones detected an N676K mutation in one allele (Figure 1B). To assess the drug resistance in these clones, we conducted cell viability assays with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 72 h after FI-700 treatment. While FI-700 inhibited the cell growth of parent MOLM-13 cells even at 0.01 µM, the resistant clones were able to survive in sub-micromolar levels of FI-700 (Figure 2A,B).

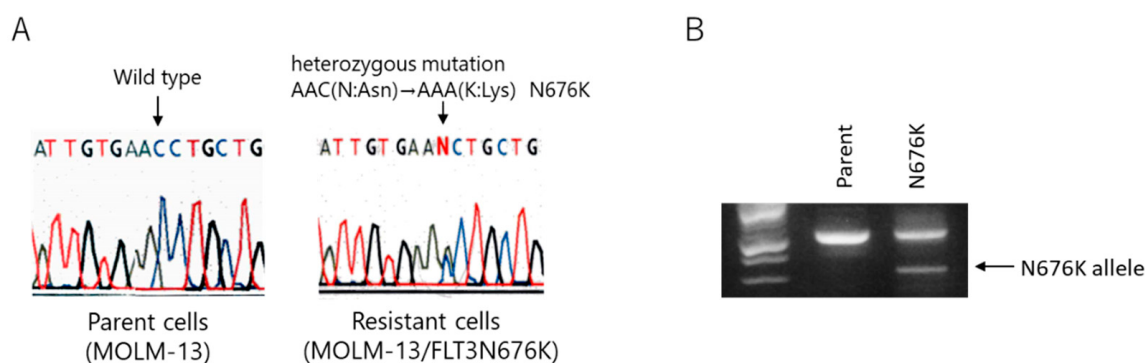


Figure 1. Establishment of MOLM-13 sublines with a resistance mutation to an FLT3 inhibitor. (A) Sanger sequencing of FLT3 in each cell line. In six of the six clones with resistance to FI-700 at more than 1 µM, the third nucleotide C of N676 was substituted with A, resulting in an Asn to Lys amino acid change (N676K). (B) An FLT3 N676K mutation was detected in one allele by allele-specific PCR, which indicated that the mutation (N676K) was identified as an acquired mutation.

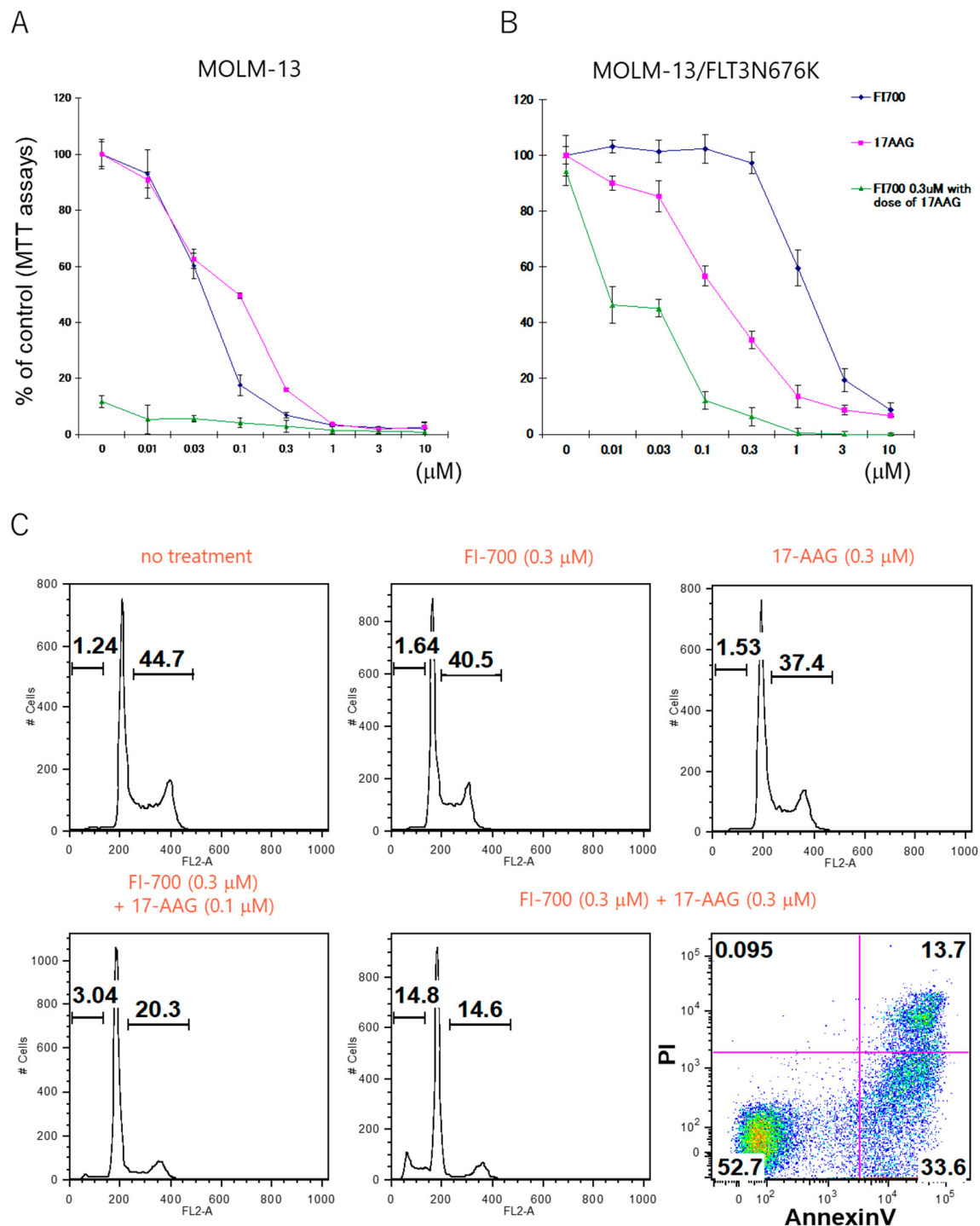


Figure 2. The combination of FI-700 and 17-AAG induced synergistic effects in MOLM-13/N676K cells. MTT assays were performed after 72 h of drug treatment. (A) In MOLM-13 cells, both the FI-700 and 17-AAG mono-treatments resulted in cell growth inhibition. (B) In MOLM-13/FLT3N676K cells, 0.3 μM FI-700 treatment did not induce any effects while 17-AAG mono-treatment did. The combination of both drugs induced synergistic effects. The data shown are the means $\pm$ standard error of the mean from three independent experiments. (C) Regarding the S/G2M fraction, the combination of FI-700 and 17-AAG induced synergistic effects in MOLM-13/N676K cells, and a higher dose of 17-AAG induced cell death, as indicated by the sub-G1 and PI-negative/positive and annexin-V fraction.

3.2. Combination Treatment of FI-700 and 17-AAG Synergistically Inhibited the Growth of Parent and FLT3 Inhibitor-Resistant MOLM-13 Cells

To assess the effect of an HSP90 inhibitor on FLT3 inhibitor-resistant AML, we treated resistant cell lines (MOLM-13/FLT3N676K) with varying concentrations of the HSP90 inhibitor 17-AAG for 72 h. MTT assay results showed that 17-AAG treatment at sub-micromolar levels inhibited the growth of both parent and resistant cell lines (Figure 2A,B). Furthermore, the combination of FI-700 and 17-AAG synergistically enhanced the growth inhibition; doses of 17-AAG lower than those used for mono-treatment in combination with 0.3 μ M FI-700 more strongly inhibited the cell growth of not only parent MOLM-13 cells but also MOLM-13/FLT3N676K cells when compared to the mono-treatment (Figure 2A,B). DNA histograms and propidium iodide (PI)/Annexin-V assays detected the induction of cell death by 17-AAG, and they also revealed the synergistic effect of the combination treatment (Figure 2C).

3.3. Combination of FI-700 and 17-AAG Inhibited FLT3 Signaling in FLT3 Inhibitor-Resistant MOLM-13 Cells

We assessed the phosphorylation levels of FLT3 and its downstream signaling proteins. Western blotting of MOLM-13/N676K substrates demonstrated that FI-700 or 17-AAG mono-treatment inhibited the phosphorylation of FLT3 and STAT5, which is an FLT3 direct downstream signaling protein, in a concentration-dependent manner. Furthermore, the combination of FI-700 and 17-AAG inhibited the phosphorylation of FLT3 and STAT5 more strongly than the mono-treatments. Of note, 17-AAG mono-treatment did not inhibit the expression of STAT5 (Figure 3A).

Unlike the results of phosphorylated FLT3, Western blotting showed that FLT3 expression in MOLM-13/N676K cells was increased by FI-700 mono-treatment. Similarly, flow cytometry analysis revealed that with FI-700 mono-treatment the expression of FLT3 on the cell surface increased in a dose-dependent manner in both parent MOLM-13 and MOLM-13/FLT3N676K cells (Figure 3C). In contrast, 17-AAG mono-treatment and the combination treatment decreased the expression level of FLT3, and the Western blotting results also showed the same findings (Figure 3A).

The phosphorylation of MAPK and AKT, which are also FLT3 downstream signaling proteins, was inhibited by 17-AAG mono-treatment, but the combination treatment of FI-700 and 17-AAG showed no synergistic effect (Figure 3B). In addition, 17-AAG mono-treatment did not change the expression of HSP90.

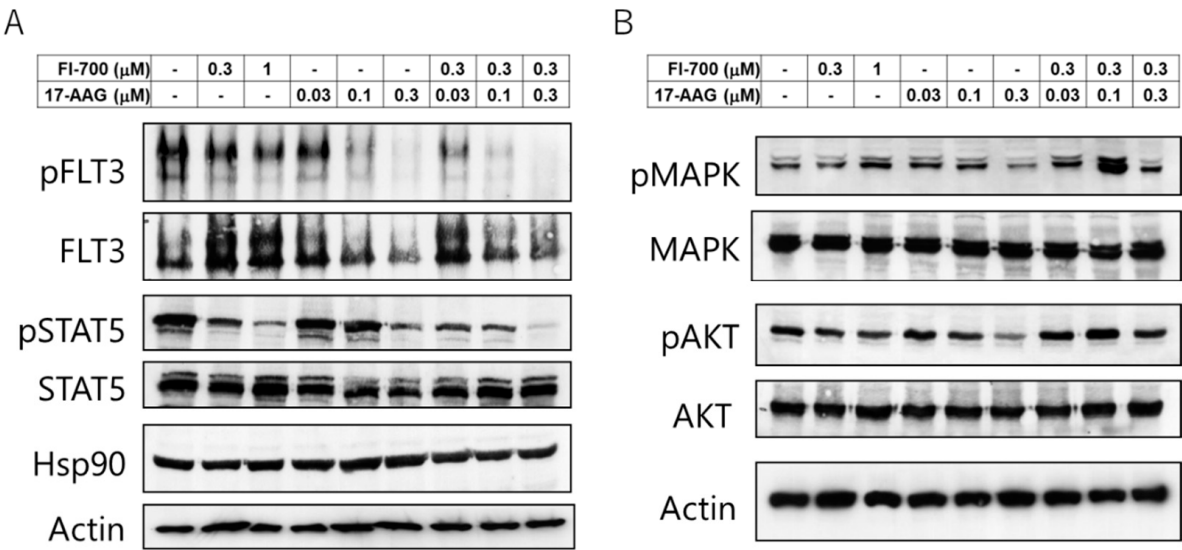


Figure 3. Cont.

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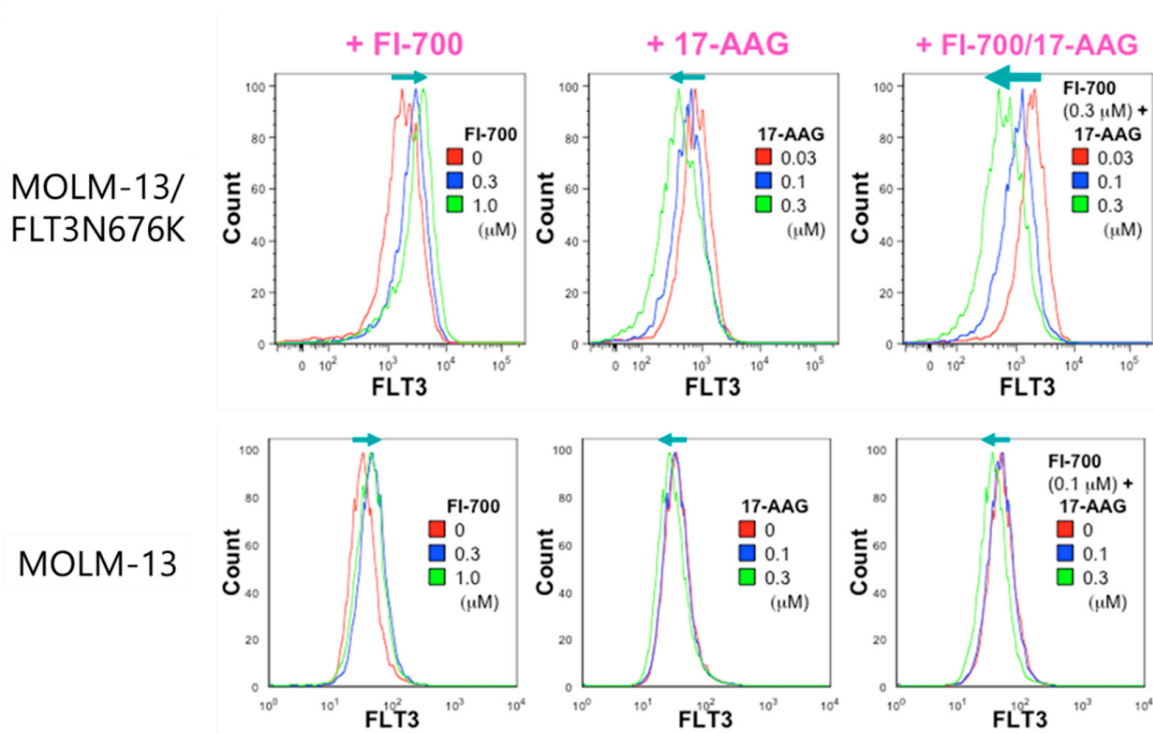


Figure 3. The expression and phosphorylation of FLT3 and its downstream signaling proteins. (A) The combination of FI-700 and 17-AAG induced synergistic effects on FLT3 and STAT5 dephosphorylation. The expression of HSP90 was not affected by any drug treatment. The expression of FLT3 increased after FI-700 treatment, while it decreased after 17-AAG treatment and after the combination treatment. (B) Phosphorylation of MAPK and AKT decreased after 17-AAG treatment. In these downstream molecules, no synergistic effect of the drug combination was seen. (C) After treatment with FI-700 for 6 h, the expression level of FLT3 increased in a dose-dependent manner. After 17-AAG treatment and after the combination treatment, the expression of FLT3 decreased, which was comparable with the Western blotting results.

3.4. Combination of 17-AAG and FI-700 Inhibited the Association of HSP90 and FLT3

To clarify the mechanism underlying the inhibitory effect of 17-AAG, we conducted immunoprecipitation and immunoblotting of HSP90 and FLT3 at 6 h after inhibitor treatment. As shown in Figure 4, FLT3 appeared to be associated with HSP90 in untreated cells of both cell lines. In MOLM-13/FLT3N676K cells, 17-AAG inhibited the association between FLT3 and HSP90, and it induced the degradation of FLT3 in a dose-dependent manner. Furthermore, the combination of FI-700 and 17-AAG significantly enhanced the dissociation of FLT3 and HSP90 in MOLM-13/FLT3N676K cells. These results suggest that disruption of the interaction between FLT3 and HSP90 by 17-AAG may lead to the reduction in phosphorylation and the degradation of FLT3, thereby leading the cell growth inhibition.

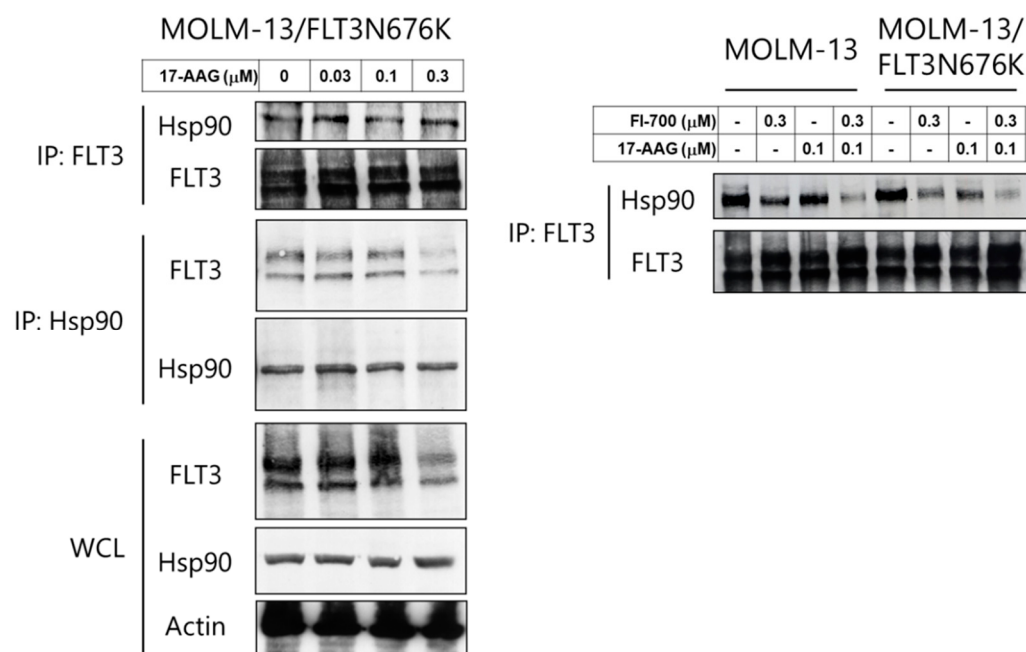


Figure 4. Immunoprecipitation and immunoblotting of FLT3 and HSP90. The association between FLT3 and Hsp90 was detected in untreated cells. 17-AAG inhibited the association between FLT3 and HSP90. The combination of FI-700 and 17-AAG enhanced the dissociation of FLT3 and HSP90.

4. Discussion

AML with FLT3-ITD is classified in the intermediate-risk in the last ELN recommendations due to the development of FLT3 inhibitors, but resistant cases with the inhibitors are still considered poor prognosis [17]. The present study provided evidence that treatment of FLT3-ITD-positive AML cells with an HSP90 inhibitor may be a promising strategy for overcoming FLT3 inhibitor resistance due to additional FLT3 mutations.

We and other groups have previously revealed that wild-type FLT3 and FLT3 mutated proteins are client proteins of HSP90 [18–21]. Moreover, cells expressing FLT3 mutants were sensitive to HSP90 inhibitors due to the inhibition of the association between FLT3 and HSP90 and the induction of FLT3 degradation [21–24]. In AML cases with FLT3 mutations, on-target resistance resulted from frequently acquiring secondary resistance mutation at the D835 residue, Y842 residue, or the gatekeeper residue F691 in the kinase domain, a well-known mechanism for resistance to FLT3 inhibitors [25]. In previous reports, N676K mutations showed leukemogenic potency and conferred resistance to FLT3 inhibitors [26,27]. FLT3-N676K alone has shown sensitivity to midostaurin, gilteritinib and quizartinib in vitro and in vivo [26,28]. However, a concurrent FLT3-ITD with N676K confers resistance to midostaurin and quizartinib [26]. In accordance with these reports, the N676K mutation in our study, which was thought to contribute to FI-700 resistance, also showed sensitivity to the HSP90 inhibitor. Thus, our present results suggest that HSP90 inhibitors can overcome this resistance mechanism. Furthermore, it has been reported that cell lines harboring the above-mentioned mutations with FLT3-TKD, such as D835Y and F691L, also confer sensitivity to the HSP90 inhibitor [21].

In the present study, we revealed the synergistic effects of an HSP90 inhibitor and FLT3-selective inhibitor. The synergistic effects of HSP90 inhibitors in combination with other anticancer treatments, such as chemotherapy, radiotherapy, immunotherapy, proteasome inhibitors, and histone deacetylase inhibitors, have already been reported [29]. HSP90 inhibitors can directly downregulate the pathways associated with drug-resistance mechanisms and indirectly enhance anticancer activity by inhibiting multiple tumor survival/growth pathways. Although the synergistic mechanism seen in our study remains unclear, we found that the combination treatment not only inhibited the phosphorylation of FLT3 and its downstream signaling proteins, but it also downregulated FLT3 on the

cell surface. While we have not performed any in vivo studies with these treatments, the synergistic effect of combination treatment requires a lower dose of each drug than monotherapy, which may reduce the likelihood of undesired side effects.

Genome profiling using next-generation sequencing has detected several common gene mutations and rearrangements in AML [6,30–32]. Although these alterations are thought to be responsible for the pathogenesis of AML and advance disease progression, the detection rate of actionable mutations has been low due to the lack of currently available targeted therapies [32]. However, a few HSP90 client proteins, such as FLT3, KIT, TP53, WT1, NRAS, and KRAS, are recurrently detected. Notably, KIT mutations are often detected in AML, systemic mastocytosis, extranodal NK/T cell lymphoma, and seminomas [33]. In AML with *RUNX1-RUNX1T1*, the presence of some parts of the *KIT* mutation, especially those in exon 17, are associated with a poor prognosis [34]. HSP90 inhibitors could suppress the growth of AML cells harboring *KIT* mutations [35,36]. In addition, gastrointestinal stromal tumors, in which *KIT* mutations are recognized as a key driver, are sensitive to HSP90 inhibitors [37]. Based on these findings, clinical trials of pimitespib, an oral HSP90 selective inhibitor, were conducted, and pimitespib was finally approved for previously treated advanced gastrointestinal stromal tumors in Japan [38]. Taking these reports into consideration, the mutations in AML described above are also potential actionable molecular targets of the HSP90 inhibitor. Actually, in mouse xenograft model with an AML cell line harboring FLT3-ITD, pimitespib exhibited potent antitumor activity [39].

In summary, our findings indicate that use of HSP90 inhibitors as a promising clinical strategy for FLT3 inhibitor-resistant AML. Further analyses, including in vivo studies and clinical trials using HSP90 inhibitors, are expected.

Author Contributions: Y.M. was the chief investigator in the trial. H.O. and Y.M. designed the research, performed the experiments, analyzed the data, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: Y.M.: Bristol-Myers Squibb, Novartis Pharma KK, Pfizer Japan, Inc., and Takeda (honoraria).

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