A Novel FLT3 Inhibitor FI-700 Selectively Suppresses the Growth of Leukemia Cells with FLT3 Mutations

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Abstract

Purpose: The aim of this study was to evaluate the antileukemia activity of a novel FLT3 kinase inhibitor, FI-700.

Experimental Design: The antileukemia activity of FI-700 was evaluated in human leukemia cell lines, mutant or wild-type (Wt)-FLT3–expressing mouse myeloid precursor cell line, 32D and primary acute myeloid leukemia cells, and in xenograft or syngeneic mouse leukemia models.

Results: FI-700 showed a potent IC50 value against FLT3 kinase at 20 nmol/L in an in vitro kinase assay. FI-700 showed selective growth inhibition against mutant FLT3-expressing leukemia cell lines and primary acute myeloid leukemia cells, whereas it did not affect the FLT3 ligand (FL)–driven growth of Wt-FLT3–expressing cells. These antileukemia activities were induced by the significant dephosphorylation of mutant FLT3 and STAT5, which resulted in G1 arrest of the cell cycle. Oral administration of FI-700 induced the regression of tumors in a s.c. tumor xenograft model and increased the survival of mice in an i.v. transplanted model. Furthermore, FI-700 treatment eradicated FLT3/ITD–expressing leukemia cells, both in the peripheral blood and in the bone marrow. In this experimental, the depletion of FLT3/ITD–expressing cells by FI-700 was more significant than that of Ara-C, whereas bone marrow suppression by FI-700 was lower than that by Ara-C.

Conclusions: FI-700 is a novel and potent FLT3 inhibitor with promising antileukemia activity.
could conquer the adverse effects of FLT3 mutations (14). Therefore, mutated or overexpressed FLT3, which leads to constitutive active kinase, serves as an important molecular target in the treatment of leukemia (15, 16). To date, several inhibitors, such as PKC412, CEP-701, MLN-518, or SU-11248 have been subjected to clinical trials in several hematologic malignancies including AML (17–20). However, the clinical efficacy of these FLT3 inhibitors in patients with AML seems unimpressive, possibly because of the potency, adverse events, or protein binding of each drug (21, 22). In addition, most of these kinase inhibitors, which are currently in development, were not originally screened for sensitivity and selectivity against the activated FLT3 kinase. Therefore, the discovery and clinical development of second generation novel FLT3 inhibitors is required. In this study, we evaluated the effects of FI-700, a novel FLT3 kinase inhibitor, on leukemia cells both in vitro and in vivo.

**Materials and Methods**

**Drugs.** Drugs such as FI-700 and MLN-518 were synthesized at the Pharmaceutical Research Center of Kyowa Hakko Kogyo. Ara-C (cytarabine) was purchased from Sankyo Pharmaceuticals. Drugs were prepared as a 10 mmol/L DMSO solution and stored at -20°C until use and freshly diluted with cell culture medium.

**Kinase assays for FI-700.** To evaluate the kinase inhibitory activities of FI-700 against FLT3 in a cell-free system, a commercially available FLT3 enzyme (recombinant human COOH-terminal 564-end amino acids; Upstate Biotechnology) and its substrate biotinylated poly-(Glu/Tyr 4:1) substrate (CISbio International) were used. FI-700 inhibition of substrate phosphorylation by a series of tyrosine and serine/threonine kinases was analyzed as previously described (23).

**Cell lines and cell culture.** Human leukemia cell line, MOLM-13, was obtained from DSMZ (German Resource Centre for Biological Material); MV4;11, THP1, K562, and HL60 were from the American Type Culture Collection; Kasumi-1 was from Hiroshima University; whereas MegO1 was established at Nagoya University. MV4;11 was maintained in Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with 10% FCS, and the other human leukemia cell lines were in RPMI 1640 supplemented with 10% FCS. It was found that MOLM-13 and MV4;11 have FLT3/ITD, THP1 expresses wild-type (Wt)-FLT3 protein on their surfaces, K562 and MegO1 have a BCR/ABL translocation, Kasumi-1 has a RUNX1/MTG8 translocation and a c-KIT mutation, and that HL60 does not express FLT3 protein. A murine IL-3–dependent myeloid progenitor cell line, 32D, was obtained from the RIKEN Cell Bank, and maintained in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Life Technologies) and 1 ng/mL murine IL-3 (R&D Systems). Wt-FLT3-, FLT3/ITD-, and FLT3/D835Y–expressing 32D cells were previously reported (7, 24, 25). Wt-FLT3–expressing 32D cells were maintained in

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**Fig. 1.** Effects of FI-700 on cell growth and mutant FLT3-mediated signals. A, chemical structure of FI-700. B, growth inhibition effect on human leukemia cell lines was evaluated by measuring viable cells after treatment with FI-700 for 72 h. FI-700 selectively and sensitively inhibits the growth of FLT3/ITD–carrying leukemia cell lines MV4;11 and MOLM3 with IC₅₀ values of 0.014 and 0.016 μmol/L, respectively. FI-700 is also potent against Kasumi-1, whereas its growth-inhibitory activity was ~10 times lower than that against MV4;11 or MOLM13. C, MOLM3 cells were treated with increasing concentrations of FI-700 for 6 h. Phosphorylation status of FLT3, STAT5, and MAPK were examined by Western blot analysis. FI-700 dose-dependently inhibits the constitutive phosphorylation of mutant FLT3 as well as its downstream molecules STAT5 and MAPK. D, MOLM3 cells were treated with increasing concentrations of FI-700 for 24 h. After treatment, the cells showed an increase in the percentage of G₁ cells and a reciprocal reduction in the percentage of the S phase. Apparent increase of sub-G₁ apoptotic cells was also observed at 0.10 μmol/L of FI-700.
RPMI 1640 supplemented with 10% FCS and 1 ng/mL of murine IL-3. FLT3/ITD- and FLT3/D835Y–expressing 32D cells were maintained in RPMI 1640 supplemented with 10% FCS without IL-3. Furthermore, we cloned human FLT3/ITD cDNA into the pMX–internal ribosomal entry site–green fluorescent protein (GFP) vector (kindly provided by Professor Toshio Kitamura, Tokyo University, Japan), transduced into 32D cells as previously described, and established stable FLT3/ITD-GFP–expressing 32D cells (FLT3/ITD-GFP-32D). FLT3/ITD-GFP-32D cells revealed autonomous proliferation at the same level as FLT3/ITD- and FLT3/D835Y–expressing 32D cells, and were maintained in RPMI 1640 supplemented with 10% FCS without IL-3.

**Primary patient samples.** Bone marrow (BM) samples from patients with AML were subjected to Ficoll-Hypaque (Pharmacia LKB) density gradient centrifugation. All samples were morphologically confirmed to contain >90% leukemia cells after centrifugation on May–Grunwald Giemsa–stained cytospin slides, and then cryopreserved in liquid nitrogen before use. Informed consent was obtained from all patients for the use of their samples for the present study, as well as banking and molecular analysis. Approval for this study was obtained from the ethical committees of Nagoya University and Ogaki Municipal Hospital. Each karyotype was determined by standard G-banding analysis. Mutations of the FLT3 gene were examined as previously reported (7, 26, 27).

**Growth inhibition and cell cycle analyses.** Cell lines and primary AML cells were suspended in RPMI 1640 (Life Technologies) containing 10% FCS, and 2 × 10^6 cells per well were seeded in 96-well culture plates with or without FI-700. Cell viability was measured using the CellTiter^{	ext{TM}} Proliferation Assay (Promega) according to the instructions of the manufacturer. These procedures were done thrice independently.

For cell cycle analysis, MOLM-13 cells (3 × 10^5) were treated with increasing concentrations of FI-700 for 24 h. DNA contents were analyzed as previously described (28).

**Western blot analysis.** Anti–phospho-MAPK and anti–ERK2 antibodies were purchased from New England Biolabs. Anti-FLT3 and anti–STAT5A antibodies were from Santa Cruz Biotechnology. Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology. Anti–phospho-STAT5 mouse monoclonal antibody was prepared in the Pharmaceutical Research Center of Kyowa Hakko Kogyo. Cell lysates were extracted, separated by SDS-PAGE, and electrophoretically blot onto Immobilon PVDF membranes (Millipore), as previously described (24). FLT3 proteins were immunoprecipitated with anti-FLT3 antibodies. The precipitated samples were subjected to immunoblot analysis to detect the tyrosine phosphorylation using an antiphosphotyrosine antibody (4G10). The membranes were incubated with stripping buffer, and then reprobed with anti-FLT3 antibody.

In vivo antileukemia effects on xenograft transplantation. Severe combined immunodeficiency (SCID) mice (Fox Chase C.B-17/Icr-scid/ci, male, 5 weeks old) were purchased from CLEA Japan. Mice were treated with an i.p. injection of anti–asialo-GM1 antibody (0.3 mg/mouse; Wako Pure Chemical Industries). The day after anti–asialo-GM1 antibody treatment, all mice were s.c. inoculated in the shaved area with 1 × 10^7 of MOLM-13. Five days after inoculation, tumor volume was measured using the Antitumor test system II (Human Life), the computer operation system with the software program and instruments. Twenty-five mice with tumors ranging from 90 to 130 mm^3 were selected and randomized using the Antitumor test system II. The selected mice were divided into five groups (five mice each), and FI-700 (100 or 200 mg/kg b.i.d.) was given orally (p.o.) for 5 days from the 7th day after inoculation. Because our preliminary study showed that the administration of 400 mg/kg b.i.d. for 5 days induced body weight loss in SCID mice, we selected this treatment schedule (data not shown). The tumor volume was measured every 2 days for 10 days. Tumor volume was calculated by the Antitumor test system II as follows: tumor volume = D_1 × D_2 × D_3/2 (D_i, long diameter; D_s, short diameter). Relative tumor volume was represented as V/V_0 (V_0, initial tumor volume; V, tumor volume after dosing). The relative V/V_0 value on each day was represented as V/V_0 of vehicle-treated control mouse; T, V/V_0 of FI-700–treated mouse. We also compared the antileukemia effects between FI-700 and other potent FLT3 inhibitors, MLN-518 and PKC-412, in the same xenograft model. The MOLM-13 inoculated SCID mice were administered with MLN-518 (160 mg/kg b.i.d.) or PKC-412 (400 mg/kg q.d.) for 5 days. These doses of MLN-518 and PKC-412 were selected as the maximum tolerated dose according to previous reports (29, 30) and our preliminary examinations. In the other xenograft model, SCID mice, treated with anti–asialo-GM1 antibody, were i.v. inoculated with 1 × 10^7 of MOLM-13 cells. Thirteen days after inoculation, mice were randomized and divided into three groups (five mice each). From the day after randomization, mice were given FI-700 at 200 mg/kg (p.o.) b.i.d., Ara-C at 75 mg/kg (i.v.) b.i.d., or vehicle for 3 days. In our preliminary study, the i.v. administration of Ara-C at 150 mg/kg q.d. for 5 days induced severe body weight loss and occasionally (not always) killed animals in leukemia-transplanted status, whereas that of Ara-C at 75 mg/kg b.i.d. for 5 days was tolerable. We, therefore, decided that 75 mg/kg/d was the maximum tolerated dose in our experiment model.

In vivo antileukemia effects on syngeneic transplantation. C3H/Hej mice were purchased from Charles River Japan. Fifteen mice were i.v. inoculated with 2 × 10^7 of FLT3/ITD-GFP-32D cells, then randomly divided into three groups (five mice each). From the 7th day after inoculation, peripheral blood (PB) was collected from the mice. From the 10th day after inoculation, mice were given FI-700 at 200 mg/kg (p.o.) b.i.d., Ara-C at 150 mg/kg (i.v.) q.d., or vehicle for 4 days. Six hours after the last administration, PB were collected. Total RNA was extracted from each PB using a QIAamp RNA Blood Mini Kit (Qiagen).
Inc.). cDNA was synthesized from each RNA using a random primer and Moloney murine leukemia virus reverse transcriptase (Super-Script II; Life Technologies) according to the manufacturer’s recommendations. The expression level of the human FLT3 transcript was quantitated using a real-time fluorescence detection method on an ABI Prism 7000 sequence detection system (Applied Biosystems) as previously reported (13, 31). After the collection of PB, mice were sacrificed, and then femora and spleens were collected. BM cells were collected from femora and the total cell number from each femur was counted using a cell counter. In order to discriminate the FLT3/ITD-GFP-32D leukemia cells and normal BM cells, all collected cells were subjected to flow cytometry analysis after PE-conjugated anti-human FLT3 monoclonal antibody (DAKO) staining. In this flow cytometry analysis, GFP-positive cells were defined as residual leukemia in the femur. The weight of each collected spleen was measured.

Statistical analysis. Differences in continuous variables were analyzed with the unpaired t test for distribution between two groups. Survival probabilities were estimated by the Kaplan-Meier method, and differences in survival distributions were evaluated using the log-rank test. Differences in therapeutic effects were analyzed with the repeated-measures ANOVA method. These statistical analyses were done with StatView-J 5.0 (Abacus Concepts Inc.). For all analyses, the P values were two-tailed, and P < 0.05 was considered statistically significant.

Results

Selective kinase inhibition by FI-700. A novel small-molecule FLT3 kinase inhibitor, FI-700, was identified by screening the chemical libraries of Kyowa Hakko Kogyo. The chemical structure of FI-700 is shown in Fig. 1A. FI-700 inhibited FLT3 kinase with an IC50 of 0.02 μmol/L for in vitro kinase assays. The selectivity of FI-700 was examined against a wide range of kinases (Table 1). Although FI-700 at 1 μmol/L showed >50% inhibition against KIT, FMS, or LYN kinases, it did not possess potent kinase inhibitory activity against ALK, TRKB, ABL, EGFR, FES, TIE2, MEK1 or CDK2.

Growth-inhibitory effects. To evaluate the selectivity and sensitivity of FI-700 in the cellular system, we examined its growth-inhibitory effects on FLT3-transfected 32D cells as well as on several human leukemia cell lines. Cells were treated with increasing concentrations of FI-700 for 72 h, and then viable cells were determined by the CellTiter96 Proliferation Assay. FI-700 selectively inhibited the growth of FLT3/ITD- and FLT3/D835Y-expressing 32D cells with IC50 values of 0.057 and 0.070 μmol/L, respectively, but not that of parental 32D cells with 1 ng/mL of IL-3 (Table 1). Notably, the IC50 value against WT-FLT3-expressing 32D cells with 100 ng/mL of FL was more than 10 times lower than that against mutant FLT3-expressing 32D cells. Furthermore, growth inhibition against FLT3/ITD- and FLT3/D835Y-expressing 32D cells was cancelled by the addition of IL-3, indicating the selective inhibitory profile of FI-700 against constitutively active FLT3 kinases.

Consistent with the results from mutant FLT3-expressing 32D cells, FI-700 inhibited the growth of human leukemia cell lines harboring FLT3/ITD in a dose-dependent manner (Fig. 1B). As shown in Table 1, the IC50 values of FI-700 against MV4;11 and MOLM-13 were 0.014 and 0.016 μmol/L, respectively, whereas those against WT-FLT3 expressing human leukemia were 10- to 100-fold higher. The IC50 value against Kasumi-1, which has an activating mutation in the cKIT gene, was 0.18 μmol/L, indicating the inhibitory potency of FI-700 against mutant cKIT kinase. In addition, the presence of FL did not affect the sensitivity of FI-700 against all human leukemia cell lines regardless of the expression of FLT3 protein (data not shown).

Inhibitory effects of FI-700 on FLT3 and downstream signals. MOLM13 cells were treated with increasing concentrations of FI-700 for 6 h. Cell lysates were subjected to Western blot analysis to detect the phosphorylation status of FLT3, signal transducers and activators of transcription 5 (STAT5), and mitogen-activated protein kinase (MAPK). FI-700 suppressed
the autophosphorylation of FLT3 as well as downstream molecules, such as STAT5 and MAPK, in a dose-dependent manner (Fig. 1C). Dephosphorylation of FLT3, STAT5, and MAPK was observed at concentrations above the IC_{50} value against MOLM-13 cells. Similar down-regulation of phosphorylated FLT3 and STAT5 was also observed in MV4;11, FLT3/ITD–, and FLT3/D835Y–expressing 32D cells (data not shown). These results indicated that FI-700 inhibited the growth of mutant FLT3-expressing cells by the dephosphorylation of constitutively active FLT3 kinase.

**Cell cycle effects of FI-700.** After treatment with increasing concentrations of FI-700 for 24 h, MOLM-13 cells exhibited an increase in the percentages of G_1 cells in comparison with controls. Simultaneously, a reciprocal reduction in the percentage of cells in the S-G_2 phase was observed. An apparent increase of sub-G_1 apoptotic cells was also observed at >0.10 \mu M of FI-700 (Fig. 1D). These results indicated that dephosphorylation of FLT3 by FI-700 could induce cell cycle arrest and eventually cause apoptosis at concentrations above the IC_{50} value against MOLM-13 cells.

**Antileukemia effect of FI-700 against s.c. MOLM-13 tumor xenografts in SCID mice.** MOLM13 cells (1 × 10^7/mouse) were s.c. inoculated into SCID mice on day -6. The mean initial tumor volume on day 0 (V_0) was 149.2 ± 22.8 mm^3. The mice were orally given vehicle or FI-700. The ratio of tumor volume in the treated to control mice (T/C) and the relative tumor volume to V_0 (V/V_0) in each group were evaluated until day 10. In the MOLM-13 tumor xenograft model, oral administration of FI-700 at 100 or 200 mg/kg b.i.d. for 5 days showed a potent and significant antitumor effect in a dose-dependent manner (Fig. 2A). FI-700 dosing at 100 and 200 mg/kg showed growth inhibition of tumors with T/C minimum values (T/C_{min}) of 0.10 (on day 7) and 0.006 (on day 10), respectively (Fig. 2B). In addition, FI-700 dosing showed tumor regression, giving V/V_0 (0.571) on day 7 at 100 mg/kg and V/V_0 (0.098) on day 10 at 200 mg/kg, respectively. Although the regrowth of MOLM-13 tumors was observed at 100 mg/kg after stopping FI-700 treatment, continuous regression of tumors was observed in mice treated with 200 mg/kg until day 10. No significant suppression of body weight gain or mortality was observed in the FI-700–treated group during the experiments. T/C_{min} values of MLN-518 and PKC-415 were 0.45 and 0.20 on day 7, respectively. However, tumor regression was not observed in either MLN-518- or PKC-412–treated mice.

**Table 2. Treatment groups and dosing schedules used.**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Schedule</th>
<th>T/C_{min} (on day)</th>
<th>Regression V/V_0_{min} (on day)</th>
<th>Body weight loss, g (on day)</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>1.00</td>
<td></td>
<td></td>
<td>0/5</td>
</tr>
<tr>
<td>FI-700</td>
<td>100</td>
<td>p.o.</td>
<td>b.i.d. × 5</td>
<td>0.10 (7)</td>
<td>0.571 (4)</td>
<td>0.7 (4, 7)</td>
</tr>
<tr>
<td>MLN-518</td>
<td>200</td>
<td>p.o.</td>
<td>b.i.d. × 5</td>
<td>0.006 (10)</td>
<td>0.098 (10)</td>
<td>--</td>
</tr>
<tr>
<td>PKC-412</td>
<td>160</td>
<td>p.o.</td>
<td>q.d. × 5</td>
<td>0.48 (7)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PKC-412</td>
<td>400</td>
<td>p.o.</td>
<td>q.d. × 5</td>
<td>0.20 (7)</td>
<td>--</td>
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</tr>
</tbody>
</table>

NOTE: FI-700 dosing at 100 and 200 mg/kg showed growth inhibition of tumors with T/C_{min} values of 0.10 (on day 7) and 0.006 (on day 10), respectively. Although the regrowth of MOLM-13 tumors was observed at 100 mg/kg after stopping FI-700 treatment, continuous regression of tumors was observed in mice treated with 200 mg/kg until day 10. No significant suppression of body weight gain or mortality was observed in the FI-700–treated group during the experiments. T/C_{min} values of MLN-518 and PKC-415 were 0.45 and 0.20 on day 7, respectively. However, tumor regression was not observed in either MLN-518- or PKC-412–treated mice.

However, no tumor regression was observed both in MLN-518- or PKC-412–treated animals (Table 2).

In the other xenograft model, in which MOLM-13 cells were i.v. inoculated into SCCID mice, FI-700–treated mice showed a significantly longer survival time than Ara-C–treated mice (P = 0.019, log-rank test; Fig. 3).

**Inhibitory effects of FI-700 on leukemia cells in a syngeneic transplantation model.** FLT3/ITD-GFP-32D cells were i.v. inoculated into syngeneic C57H1/Hej mice, and FI-700, Ara-C, or vehicle was administered to the mice 11 days after inoculation. In this model, the in vivo efficacy of FI-700 against FLT3/ITD-expressing cells was compared with the conventional antileukemic agent Ara-C (Fig. 4A). By quantitating the human FLT3 transcripts, we first compared the number of FLT3/ITD-GFP-32D cells in PB before and after treatment with each drug. At the 7th day after inoculation, mean FLT3 transcript levels were 24.4 ± 6.7, 157.3 ± 139.4, and 42 ± 12.5 copy/μgRNA in vehicle-, FI-700-, and Ara-C–treated mice, respectively. In all vehicle-treated mice, FLT3 transcript levels increased, and the mean FLT3 transcript level on day 13 was significantly suppressed with FI-700 treatment (P = 0.019, log-rank test).
5,869.6 ± 1,640 copy/μgRNA. In contrast, FI-700 treatment repressed the expansion of FLT3/ITD-GFP-32D cells as the decrease of FLT3 transcript levels was observed in all mice, and the mean FLT3 transcript level was 13.5 ± 10.6 copy/μgRNA on day 13. The increased level of FLT3 transcripts in all Ara-C–treated mice was lower than that in vehicle-treated mice, although the effect of Ara-C treatment was limited as the mean FLT3 transcript level was 882.7 ± 305.5 copy/μgRNA on day 13. These results showed that the repressive effects of FI-700 on the expansion of FLT3/ITD-GFP-32D cells were significantly stronger than those by Ara-C (P = 0.0263, repeated-measures ANOVA; Fig. 4B). We next compared the percentage of residual

### Table 3. Clinical characteristics of each case

<table>
<thead>
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<th>Case</th>
<th>French-American-British classification</th>
<th>Age (y)</th>
<th>Karyotype</th>
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</thead>
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<tr>
<td>Wt-1</td>
<td>M2</td>
<td>55</td>
<td>46,XX, t(8;21)(q22;q22)</td>
</tr>
<tr>
<td>Wt-2</td>
<td>M3</td>
<td>54</td>
<td>46,XY</td>
</tr>
<tr>
<td>KDM</td>
<td>M2</td>
<td>41</td>
<td>46,XX, t(15;17)(q22;q21)</td>
</tr>
<tr>
<td>ITD-1</td>
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<td>46,XX</td>
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<tr>
<td>ITD-2</td>
<td>M1</td>
<td>50</td>
<td>46,XY</td>
</tr>
</tbody>
</table>
BM FLT3/ITD-GFP-32D cells in the femur between FI-700 and Ara-C treatment using flow cytometry analysis. As shown in Fig. 4B, the mean percentage of FLT3/ITD-GFP-32D cells in BM was 20.2 ± 3.4% in vehicle-treated mice. In contrast, the mean percentage of FLT3/ITD-GFP-32D cells was decreased to 8.0 ± 2.4% in the FI-700–treated group, whereas that of Ara-C–treated mice was 31.6 ± 1.6% (P = 0.0143; Fig. 4B). Notably, the total number of nucleated cells in the BM of Ara-C–treated mice was significantly decreased when compared with that of FI-700–treated mice [(0.42 ± 0.06) × 10⁷ and (1.19 ± 0.06) × 10⁷ cells/femur, respectively, P < 0.0001], whereas the total cell number of FI-700–treated mice was the same as that of healthy control mice [(1.53 ± 0.23) × 10⁷ cells/femur, Fig. 4C]. Furthermore, the mean spleen weight of FI-700–treated mice (67.1 ± 4.9 mg) was significantly lighter than that of Ara-C- or vehicle–treated mice (122.2 ± 9.1 mg; P = 0.0017; 249.7 ± 33.9; P = 0.0021, respectively; Fig. 4D). These results indicate that FI-700 has the potential to eradicate leukemia cells harboring FLT3 mutations without severe BM suppression.

**Growth-inhibitory effects on primary AML cells.** We finally analyzed the in vitro growth-inhibitory effects of FI-700 on five primary AML cells consisting of two Wt-FLT3, two FLT3/ITD, and one FLT3/D835Y case. The characteristics of these AML cases are shown in Table 3. Because the viability of primary AML cells drastically decreased even when cultured without FI-700 for >3 days, we evaluated the viable AML cells after treatment with or without 100 nmol/L of FI-700 for 48 h. FI-700 did not affect the growth of two AML cells with Wt-FLT3, whereas it reduced the growth of all FLT3/ITD- or FLT3/D835Y-AML cells (Fig. 5). Furthermore, the addition of FL (100 ng/mL) did not affect the growth-inhibitory activity of FI-700 against primary AML cells regardless of FLT3 mutations (Fig. 5).

**Discussion**

FLT3 kinase is thought to be a promising therapeutic target for acute leukemia, and it is highly expected that the development of FLT3-selective small-molecule tyrosine kinase inhibitors will make a more efficacious therapeutic strategy for leukemia therapy. To date, several tyrosine kinase inhibitors have been shown to have the potency to inhibit the FLT3 kinase, whereas no FLT3 kinase–specific inhibitor has been approved as an antileukemia agent. FI-700 has been discovered and developed as an orally available and selective kinase inhibitor against FLT3. In this study, we evaluated the antileukemia activity and proof of concept of FI-700 as a FLT3 kinase inhibitor both in vitro and in vivo.

FI-700 showed potent and selective inhibitory activity against FLT3 kinase by in vitro kinase assays. In addition, it showed little inhibitory activity against a wide variety of tyrosine and serine/threonine kinases. This kinase inhibition profile was also confirmed by the cellular system as potent and selective growth inhibition against both FLT3/ITD- and FLT3/D835Y-carrying cells. It was further shown that growth inhibition by FI-700 was dependent on the dephosphorylation of constitutively active mutant FLT3 kinases as well as STAT5 and MAPK. In addition, cell cycle analysis revealed that FI-700 induced G₁ arrest over the concentration of IC₅₀ value against mutant FLT3-expressing leukemia cells, resulting in apoptosis. On the other hand, its potency against constitutively active FLT3 kinase was >10 times higher than Wt-FLT3 kinase. Because hematopoietic progenitors express Wt-FLT3, the lower potency against it seemed an advantage to avoid BM suppression in clinical use. These characteristics of FI-700 obtained from in vitro analyses might be favorable characteristics of FI-700 for patients with leukemia.

To evaluate the antileukemia efficacy of FI-700 in vivo, we employed two distinct s.c. or i.v. inoculated xenograft model. In the s.c. inoculated model, oral treatment of mice with FI-700 for 5 days showed tumor regression without body weight loss. In this model, whereas tumor regression was transient and tumor regrowth was observed after stopping FI-700 at 100 mg/kg, continuous tumor regression was observed in mice treated with 200 mg/kg, suggesting the curative potency of FI-700. When we compared the efficacy of FI-700 with those of MLN-518 or PKC-412 at the maximum tolerated dose, oral treatment of FI-700 showed superior antileukemia activity in the MOLM-13 inoculated model. Furthermore, in the i.v.-inoculated model, 5-day treatment with FI-700 prolonged the life span of leukemia-inoculated mice and its efficacy was more potent than that of conventional therapeutics, Ara-C. However, all mice eventually died even in the FI-700–treated group, indicating that longer treatment with FI-700 was necessary for the complete eradication of leukemia cells. Our preliminary study showed that 14-day treatment with FI-700 at 200 mg/kg b.i.d. could prolong the survival compared with 5-day treatment, suggesting that longer treatment with FI-700 might improve the efficacy of the drug. Therefore, a longer treatment schedule could be necessary to provide a cure for leukemia.

The efficacy of FI-700 was further confirmed in the other syngeneic i.v. transplanted model. In this model, we compared the potency against leukemic FLT3/ITD-expressing cells between FI-700 and Ara-C. As shown in Fig. 4, it was quantitatively shown that FI-700 significantly eradicated more FLT3/ITD-expressing cells both from PB and BM compared with Ara-C. The most important finding was that BM suppression by FI-700 was significantly less than that by Ara-C. This lower BM suppression might depend on the selective kinase inhibition profile of FI-700. The treatment schedule of Ara-C used here almost corresponds with the high-dose Ara-C.
regimen, which is clinically used for patients with leukemia. Although it remains unclear whether high-dose chemotherapy, such as the high-dose Ara-C regimen, conquers the adverse effect of FLT3 mutations, two large-scale studies raised the possibility that therapeutic regimens employing high-dose Ara-C might contribute towards a better outcome in patients with AML (32, 33). The high-dose Ara-C regimen, a dose-intensified regimen, is an established treatment strategy both for newly diagnosed and relapsed or refractory acute leukemia patients, whereas severe BM suppression is known to be an unavoidable adverse effect of this regimen (34). In the present mice model, BM suppression was consistently induced by Ara-C treatment, although the antileukemia effect was limited. Therefore, the selective antileukemia activity of FI-700 against FLT3/ITD-expressing cells without severe BM suppression could be ideal for the treatment of patients with leukemia.

It has been suggested that AML is the consequence of two broad complementation classes of mutations: those that confer a proliferative and/or survival advantage to hematopoietic progenitors (class I) including activating mutations in tyrosine kinases or their downstream effectors, and those that impair hematopoietic differentiation and confer properties of self-renewal (class II) including rearrangements or point mutations of core-binding factor genes and PML-RARA (35). FLT3 mutations have been frequently found in AML patients with PML-RARA translocation, whereas they have also been preferentially found in AML patients with a normal karyotype, suggesting that additional genetic alterations are involved in the pathogenesis of AML with FLT3 mutations (8). Therefore, it is thought to be difficult to cure leukemia by monotherapy with a FLT3 inhibitor. This is also supported by recent results from clinical trials of potent FLT3 inhibitors, such as MLN-518, PKC-412, or CEP-701. Although FI-700 showed a growth-inhibitory effect on primary AML cells with FLT3 mutations, combination effects with conventional chemotherapeutic agents should be further evaluated.

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References


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