G-749, a novel FLT3 kinase inhibitor can overcome drug resistance for the treatment of Acute Myeloid Leukemia

Hee Kyu Lee1, Hong Woo Kim2, In Yong Lee2, Jungmi Lee2, Jaekyoo Lee2, Dong Sik Jung1, Sang Yeop Lee1, Sung Ho Park1, Haejun Hwang1, Jang-Sik Choi1, Jung-Ho Kim1, Se Won Kim1, Jung Keun Kim1, Jan Cools3, Jong Sung Koh2, and Ho-Juhn Song2*

1Oscotec Inc., Korea Bio-Park, 694-1, Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do, 463-400 Republic of Korea; 2 Genosco, 767C Concord Ave, Cambridge, MA 02138, USA; 3 Center for the Biology of Disease, VIB, Leuven, Belgium

Running Title: FLT3 inhibition by G-749

*Corresponding author:
Ho-Juhn Song, Ph.D.
767C Concord Avenue, Cambridge, MA 02138, U.S.A.

Email) hsong@genosco.com
Phone) 1-617-494-1460
Key Points

1. A novel inhibitor G-749 is very potent against FLT3 kinase mutants including D835Y and ITD/F691L that confer resistance to PKC412 and AC220.

2. G-749 shows several desirable characteristics to overcome other drug resistances conferred by patient plasma, FLT3 ligand and stromal cell.
ABSTRACT

Aberrant activations of the receptor tyrosine kinase FLT3 are implicated in the pathogenesis of 20–30% of patients with acute myeloid leukemia (AML). G-749 is a novel FLT3 inhibitor that showed potent and sustained inhibition of the FLT3 wild type and mutants including FLT3-ITD, FLT3-D835Y, FLT3-ITD/N676D, and FLT3-ITD/F691L in cellular assays. G-749 retained its inhibitory potency in various drug-resistance milieus such as patient plasma, FLT3 ligand surge, and stromal protection. Furthermore it displayed potent antileukemic activity in bone marrow blasts from AML patients regardless of FLT3 mutation status including those with little or only minor responses to AC220 or PKC412. Oral administration of G-749 yielded complete tumor regression and increased lifespan in animal models. Thus, G-749 appears to be a promising next generation drug candidate for the treatment of relapsed and refractory AML patients with various FLT3-ITD/-TKD mutants and further shows ability to overcome drug resistance.

Key words: AML, FLT3-ITD, FLT3-TKD, FLT3-WT, bone marrow plasma, stromal protection, drug-resistance.
INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematological disorder in which the hematopoietic progenitor cells lose their ability to differentiate normally and continue to proliferate. Fms-like tyrosine receptor kinase-3 (FLT3) plays an important role in normal hematopoiesis and leukemogenesis and is expressed in most of AML blasts. In 20-25% of AML patients, the FLT3 gene acquires an internal tandem duplication in the juxtamembrane domain of FLT3 (FLT3-ITD) and this is associated with poor prognosis. FLT3 point mutations within the activation loop of the tyrosine kinase domain (FLT3-TKD) have also been detected in approximately 7% of AML patients. FLT3-ITD or -TKD mutants undergo constitutive autophosphorylation of FLT3, causing aberrant signaling activation of several pathways such as Ras/MAP, JAK/STAT5, and AKT. Activated FLT3-kinase mutations eventually induces transformation and tumorigenesis in hematopoietic cells and suppresses normal myeloid differentiation and therefore are attractive therapeutic targets to treat AML.

A number of receptor tyrosine kinase inhibitors (TKIs) targeting FLT3 have been developed and clinically examined in AML patients; Midostaurin (PKC412), the multiple kinase inhibitor in Phase III trials in combinational chemotherapy, Sorafenib in Phase I trials in relapsed/refractory AML patients, and Quizartinib (AC220), a selective FLT3 inhibitor in Phase II trials. PKC412 induced a 92% complete remission (CR) rates in FLT3-mutated AML in combinational chemotherapy, Sorafenib achieved a 23% CR in 65 FLT3-ITD AML patients after chemotherapy or allogeneic hematopoietic stem cell transplantation and AC220 led to a 3-6% CR and 46-54% composite CR rate in relapsed/ refractory 190 FLT3-ITD positive AML patients.

The development of drug-resistance during the treatment of hematological malignance has been a challenging issue for TKIs. Recent evidences suggest that the majority of patients treated with single FLT3 inhibitor experienced only transient and partial response due to the development of drug resistance, which hinders treatment with FLT3-TKIs. Various factors including point mutations, plasma inhibitory activity (PIA), protective effect by bone marrow stromal cells, and high levels of
FLT3 ligand (FL) have been identified to confer FLT3 inhibitors drug resistance. Point mutations within the kinase domain of FLT3-ITD especially at the position of N676, F691, and D835 lead to substantial resistance to AC220 and PKC412. Additional resistance mutations to AC220 have also been described using in vitro models. Drug resistance comes from PIA where the inhibition of PKC412 and CEP701 against FLT3 autophosphorylation was dramatically decreased in human plasma milieu. The bone marrow microenvironment also contributes to the reduction of drug sensitivity in vivo. It was shown that bone marrow stromal cells support the survival of neighboring blast cells, resulting in the long-term survival and growth of leukemia cells. The increased secretion of FLT3 ligand after induction therapy of Cytarabine was also known to attenuate efficacy of FLT3 inhibitors. Therefore, there is an unmet need for the next generation FLT3 inhibitor to overcome drug resistance.

Here, we report that a novel inhibitor G-749 with a unique kinase inhibition profile is very potent against FLT3 kinase and provides sustained inhibition of FLT3 phosphorylation and downstream effectors in FLT3-ITD expressing cell lines. Using BaF3 model cells, we demonstrate that G-749 is highly potent against clinically known FLT3 mutants including gatekeeper and TKD that confer resistance to PKC412 and AC220. Notably, in comparison with PKC412 and AC220, G-749 shows several desirable characteristics to overcome other known drug resistances conferred by patient plasma, FL surge, and protection by stromal cells. Oral dosing of G-749 leads to complete tumor regression without relapse in the mouse xenograft model and increases survival in bone marrow engraft model. Furthermore, G-749 shows potent anti-leukemic activity in patient blasts harboring FLT3-ITD, -TKD and -ITD/TKD mutations through inhibition of p-FLT3 and p-ERK1/2, including those with little or only minor response to AC220 and/or PKC412. Thus, we believe that G-749 is a promising drug candidate with strong therapeutic potential to overcome drug resistance for AML treatment.
METHODS

Kinase assay, Cell culture, and FLT3 inhibitors

Biochemical assay for FLT3-WT and -D835Y was performed according to Perkin-Elmer TR-FRET protocol using suggested experimental materials. The detail method is described in Supplementary Method. Human leukemia cell line Molm-14 was kindly provided by Dr. S. Kang, Emory School of Medicine; MV4-11, RS4-11, K562, HEL, and HS-5 were obtained from ATCC. The six different BaF3 cell lines were kindly provided by Dr. J. Cools. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) or calf bovine serum, 2 mM of L-glutamine and 1% of the antibiotics penicillin/streptomycin and cultured in a humidified atmosphere at 37°C with 5% CO2. G-749 was synthesized at Genosco (Cambridge, MA). Its synthetic process is described in Supplementary Figure 1 and Method. The FLT3 inhibitors AC220 and PKC412 were purchased from LC Laboratories.

Cell proliferation, Apoptosis, and FACS analysis

Cells were seeded at a density of 2 X 10^4 cells/well and treated with the indicated concentrations of test inhibitor for 72 hours at 37°C. The conditioned medium (CM) was prepared from HS-5 cells culture for 5 days under routine culture conditions, clarified by centrifugation and used immediately. The CM was added to complete medium at a final concentration of 35%. In co-culture experiments, 5 X 10^4 AML blast cells were plated in 24 well plates containing 1 X 10^4 HS-5 monolayers and then cultured for at least 48 hours before the exposure of inhibitors. Cell viability was determined by an ATPLite assay (Perkin-Elmer). Caspase-3/7 activity was measured by using the Caspase-Glo 3/7 assay (Promega).

For flow cytometry analysis experiments, MV4-11 cells were washed and resuspended in binding
buffer containing FITC-conjugated anti-Annexin V antibody (Roche Diagnostics). Cells were stained with APC-conjugated anti-CD45 antibody (Becton Dickinson) to exclude contamination of HS-5 stromal cells. CD45 positive cells were gated and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).

Western Blot analysis and ELISA

The phospho-FLT3 ELISA kit was purchased from Cell Signaling Technology and performed according to manufacturer’s protocol. The detail protocol of Western analysis is described in Supplementary Method.

Patient Samples

In accordance with the Declaration of Helsinki, bone marrow mononuclear cells from AML patients were collected with informed consent. Institutional Review Board (IRB) approval was obtained from Chonnam National University Hospital (Gwangju, S. Korea) and Seoul St. Mary’s Hospital (Seoul, S. Korea) for this study. The FLT3 mutation status was determined by the diagnosis protocol of each hospital organization. Mutations of the FLT3 gene were examined as previously reported.

Cryopreserved samples were thawed and incubated with culture medium enriched to 20% FBS for 90 minutes before experimental procedures. Only samples with >70% viability were used for proceeding test. For blast viability, the blasts were treated with FLT3 inhibitors for 72 hours, supplemented with 50 ng/mL IL-3 and 50 ng/mL SCF. Their viability was determined by an ATPLite assay.

In vivo mouse models

The subcutaneous MV4-11 tumor model and the bone marrow engrafted model are described in Supplementary Method. For statistical analysis, Analysis of variance (ANOVA) was performed by Prism 5.0 to examine statistical difference (detail in Supplementary Method).
RESULTS

Biochemical properties and selectivity of G-749

A novel small molecule FLT3 kinase inhibitor, G-749 was designed and synthesized using structure-based drug design approach (Figure 1A; Supplemental Figure 1). It displayed potent inhibition of FLT3 wild type (-WT) and FLT3-D835Y mutants with biochemical IC\textsubscript{50} value of 0.4 and 0.6 nM, respectively (Table 1). Even when the ATP concentration was gradually increased up to $\geq 1$ mM (Supplemental Figure 2), G-749 still retained strong inhibitory activity with low nanomolar IC\textsubscript{50} value (0.4 to 2.7 nM). These data demonstrate that G-749 is ATP competitive and shows sustained binding to the ATP binding pocket. Potent inhibition of FLT3 was confirmed in leukemia cells where G-749 inhibited autophosphorylation of FLT3 with IC\textsubscript{50} value of $\leq 8$ nM in FLT3-WT bearing RS4-11 and in FLT3-ITD harboring MV4-11 and Molm-14 cells (Figure 1C; Supplemental Figure 3A-B).

To evaluate its kinase selectivity, 100 nM G-749 was initially challenged against 282 kinases using the Millipore Kinase Profiler (Supplemental Table 2). Major kinases inhibited by G-749 were further selected to determine their IC\textsubscript{50} potency (Supplemental Table 1). This assay revealed that G-749 displayed a unique inhibition pattern. It was highly potent against FLT3, FLT3-D835Y, and Mer (1 nM of each kinase IC\textsubscript{50}). The receptor tyrosine kinases (Ret, FLT1, Axl, Fms, FGFR1, and FGFR3) and serine/threonine kinases (Aurora B and C) were also inhibited with an IC\textsubscript{50} value of 9-30 and 6-24 nM, respectively. Other kinases including c-KIT, PDGFRs, and EGFR were not potently inhibited (IC\textsubscript{50} value of $> 300$ nM); however, their mutants were significantly inhibited. Collectively, G-749 was identified as a novel and potent FLT3 inhibitor with a unique kinase inhibitory profile.

Antiproliferative activity on leukemia cells

The antiproliferative activity of G-749 was assessed in several leukemia cell lines. G-749 showed strong antiproliferation of leukemia cells addicted to FLT3-ITD (MV4-11 and Molm-14) in a dose dependent manner, whereas it did not potently inhibit proliferation of leukemia cells without FLT3
expression (HEL and K562) or of non-FLT3 addicted cells with FLT3-WT (RS4-11) (Figure 1B; Table
1). The selective and potent antiproliferative activity of G-749 was found to come from the inhibition
of FLT3-ITD because antiproliferation was rescued in FLT3-ITD addicted BaF3 cells in the presence
of IL-3 (Supplemental Figure 5). The phosphorylation of downstream effectors in the FLT3 signaling
pathway such as p-STAT5, p-AKT, p-ERK1/2, and p-FoxO3a was also potently inhibited by G-749
(Figure 1C). Similar inhibition of downstream effectors was seen in MV4-11 cells at levels
comparable to AC220 and PKC412 (Supplemental Figure 3C). G-749 treatment led to significantly
increased active caspase 3/7 level and cleaved PARP in a dose-dependent manner in both cell lines
(Figure 1F; Supplemental Figure 3D). FACS analysis revealed that G-749 treatment increased
apoptosis of MV4-11 cells in a dose dependent manner (Figure 1G). Taken together, it causes
antiproliferative activity through apoptosis.

We next investigated the sustained potency of FLT3 inhibitors. After washout, G-749 sustained strong
inhibition of p-FLT3, p-ERK and p-STAT5 in a time dependent manner while in comparison, AC220
and PKC412 gradually lost their inhibitory activity over 24 hrs (Figure 1D, 1E). Remarkably, the
potent inhibition of G-749 against FLT3 pathways sustained after washout was found to reflect
prolonged antiproliferation in comparison to AC220 and PKC412 (Supplemental Figure 5).

It was found that the antiproliferation effect of G-749 also increased synergistically when used in
combination with Cytarabine (Figure 1H; Supplemental Figure 6). Combination index calculated by
Calcusyn analysis showed good synergistic cell death in all tested concentrations (ED50: 0.58264;
ED25: 0.50659; ED50: 0.44793), suggesting that G-749 is suitable for combinatorial therapy.

**Potent inhibition of G-749 against FLT3 mutants**

To evaluate whether G-749 could inhibit several FLT3 mutants causing drug resistance, we examined
its potency against BaF3 cell lines that stably express FLT3-ITD/N676D (ITD and ATP-binding
domain mutation), -ITD/F691L (ITD and gatekeeper mutation), -D835Y (activation loop mutation) or
-D835Y/N676D (activation loop and ATP-binding mutation). While PKC412 was less potent against BaF3 cells expressing FLT3-ITD/N676D (128 nM in IC50) than those expressing FLT3-ITD/F691L or -D835Y, AC220 showed strong potency against cells expressing FLT3-ITD (3.7 nM in IC50) but significantly decreased potency against those expressing FLT3-ITD/N676D, -ITD/F691L, -D835Y and –D835Y/N676D. In comparison, G-749 showed strong potency against autophosphorylation of all tested FLT3 mutants with IC50 of < 10 nM (Figure 2A), whose inhibition level was closely correlated with antiproliferation activity (Table 1). Additionally, the direct comparison of phosphorylation levels using Western blotting revealed that G-749 more potently inhibited the autophosphorylation of the FLT3-ITD/F691L and FLT3-D835Y with IC50 of < 8 nM than PKC412 and AC220 with IC50 of 40-200 nM, respectively (Figure 2B-C).

**Effective inhibition of FLT3 pathways in high FLT3 ligand milieu**

We examined the potency of G-749 in high concentration of FL milieu. Exogenous FL addition led to elevated p-FLT3 in a dose/time dependent manner in BaF3 cells expressing FLT3-WT (Supplemental Figure 7A-B), and in RS4-11 (Supplemental Figure 3B). The increasing FL concentration in Molm-14 cells led to decreased cell death by AC220 up to about 2.5 fold and by G-749 up to about 1.9 fold (Supplemental Figure 8). These data are consistent with the hypothesis that increased FL levels could impede the efficacy of FLT3 inhibitors to some degree.

However, even at high FL level, G-749 showed more potent inhibition of p-FLT3, p-ERK1/2 and p-AKT than AC220 and PKC412 (Figure 2D-E). Notably, all tested FLT3 inhibitors potently and equally inhibited p-STAT5 in spite of high FL addition in Molm-14. FL addition did not activate p-STAT5 but slightly increased p-ERK and p-AKT levels in the BaF3 cells expressing FLT3-WT (Supplemental Figure 7B), suggesting that the FL activating p-ARK and p-ERK are responsible for slightly reducing efficacy of AC220 and G-749. Nevertheless, G-749 shows significant potency against p-FLT3, p-ERK1/2 and p-AKT in high FL milieu.
Potent inhibitory activity in normal and patient human plasma milieu

We examined the potency of G-749 in normal and patient plasma milieu to assess plasma inhibitory activity (PIA). G-749 and AC220 equally inhibited p-FLT3 in culture medium and normal human plasma milieu in a dose dependent manner but PKC412 did not inhibit p-FLT3 in plasma milieu even at the high concentration of 1 μM (Figure 3A top & middle), which was further confirmed by ELISA analysis (Supplemental Figure 9).

We further examined the potency of G-749 and AC220 by using the bone marrow plasma from 10 AML patients who achieved complete remission (CR, n=7) or relapsed (n=3) after induction therapy with Cytarabine (Supplemental Table 3). Western blot analysis revealed that G-749 consistently inhibited p-FLT3 in all 10 patient plasma milieus but AC220 significantly lost its potency with big variations even at 1 μM (p < 0.05 compared to 1 μM G-749 group, n=10) (Figure 3B), suggesting that the inhibition degree of AC220 highly varies from patient to patient. Of particular interest was the inhibition of FLT3 pathways in relapsed patient plasma milieu (Patient 10). G-749 fully inhibited p-FLT3, p-STAT5, p-AKT, and p-ERK1/2, whereas AC220 could not inhibit them even at high concentrations (Figure 3C).

To address the lost potency of AC220 in relapsed patient millieu, we examined changes in p-FLT3 in normal plasma supplemented with FL and found that AC220 showed potent inhibition of p-FLT3 (Figure 3A, bottom), thus FL in plasma does not contribute to the lost potency. In addition, the plasma protein binding of AC220 and G-749 is about 98% and 99%, respectively so that the free form of the compound is not responsible for the reduction in potency. Therefore, it is reasonable that other unknown factors in patient plasma may be involved in impeding the efficacy of FLT3 inhibitor including AC220 but excluding G-749 and remain to be addressed. Collectively, these PIA studies clearly suggest that G-749 effectively and potently inhibits FLT3 and downstream effectors in relapsed patient plasma.
Effective inhibition in stroma protecting milieu

We investigated the antileukemic activity of FLT3 inhibitors in drug-resistant milieu provided by bone marrow stromal cells known to promote leukemia cell survival\textsuperscript{22-25}. While AC220 caused only 50% cell death of MV4-11 even at high concentration of 5 \( \mu \text{M} \) in conditioned medium (CM), G-749 and PKC412 similarly caused 90% cell death at the same concentration (Figure 4A). To determine if cell death caused by FLT3 inhibitor could be attenuated in direct contact environment of stromal cell HS-5, we co-cultured MV4-11 or patient blast of genotype FLT3-ITD (patient no. 12) with HS-5 cell and then compared the potency of FLT3 inhibitors. In case of MV4-11/HS-5 co-culture (Supplemental Figure 10), the treatment of 1000 nM G-749 and PKC412 caused 93.8% and 84.5% cell death while that of 1000 nM AC220 did 60.0%. Furthermore, in co-culture of patient blast with HS-5 (Figure 4B), the treatment of 1000 nM G-749 and PKC412 caused 39.3% and 25.2% cell death while that of 1000 nM AC220 showed 16.7% similar to DMSO control. These data strongly demonstrate that G-749 shows effective potent antileukemic activity even in the stroma protecting milieu.

To better understand how FLT3 inhibitors differentially respond to the stroma protective milieu, we monitored changes in FLT3 pathway in CM milieu. G-749 and AC220 similarly inhibited p-FLT3 and p-STAT5 in CM milieu. Of particular interest was the differential inhibition of p-ERK1/2. Unlike AC220, G-749 could potently inhibit p-ERK1/2 in Molm-14 and MV4-11 (Figure 4C; Supplemental Figure 11) and its inhibition level against p-FLT3 in CM was found to be very similar to that in normal medium. G-749 showed potent inhibition against p-ERK1/2 in Molm-14 and against p-ERK1/2 and p-AKT in MV4-11 whereas AC220 showed little or no inhibition against p-ERK1/2 and/or p-AKT in both cells. Thus, the synergistically reduced inhibitory activity against p-ERK1/2 and/or p-AKT could be responsible for reduced antiproliferative activity in MV4-11 cells. Irrespective of influential and complex genetic backgrounds and protective bypass signals provided by the CM, G-749 persistently displayed potent inhibition against p-FLT3, p-ERK and p-AKT.
Effective anti-tumor activity in mouse models

To assess in vivo pharmacodynamic effect of G-749, a single dose of G-749 HCl salt (10 mg/kg) was orally administrated to subcutaneous MV4-11 xenograft mice and showed sustained inhibition of p-FLT3, p-STAT5, and p-ERK1/2 (Figure 5A-B). These data indicate that G-749 effectively inhibits FLT3 pathway and its inhibition lasts for 24 hours. Therefore, once a day oral dosing was expected to be sufficient for in vivo efficacy in the mouse model.

To examine its anti-tumor efficacy, G-749 HCl salt was orally and quaque die administrated for 28 days to the MV4-11 xenograft mice. Significant inhibition of tumor growth was observed in the 3 mg/kg/day dosing group from 4 days onward and apparent tumor regression was seen in the 10 and 30 mg/kg/day dosing group (Figure 5C). The mice dosed with 30 mg/kg/day were subsequently monitored for additional 28 days after dosing stopped to examine if the tumor grows back. There was no tumor regrowth up to 28 days suggesting complete tumor regression. Additionally, in this study, weight loss and signs of gross toxicity were not observed in any dose group (Supplemental Figure 12).

We confirmed anti-tumor efficacy in an orthogonal model of bone marrow engraftment using Molm-14 cells, which physiologically differs from the subcutaneous MV4-11 xenograft model. In engrafted NOD/SCID mice, vehicle-treated mice showed expected clinical symptoms such as hind-limb paralysis, rough coat and decreased activity and died within 9 days. But both 10 and 20 mg/kg/day dosing groups showed increased survival in dose-dependent manner (Figure 5D). Collectively, G-749 yields effective in vivo anti-tumor activity in two different leukemia animal models and is expected to yield great efficacy in clinical trials.

Potent anti-leukemia activity in blasts of AML patients

To evaluate the antileukemia activity in primary cells, bone marrow blasts were obtained from 16 AML patients diagnosed as FLT3-WT, -ITD, -D835Y, and -ITD/D835Y (Supplemental Table 4). The overall magnitude of antileukemic activity of G-749 was more effective than that of AC220 and
PKC412 (Figure 6A, Supplemental Figure 13). Remarkably, the antileukemic response of G-749 was about 75% to the blasts with FLT3-D835Y and about 50% to those with FLT3-ITD/D835Y that showed no or little response to AC220. These data are consistent with the findings from the BaF3 model cells.

We further determined whether the antileukemic activity of G-749 couples with the inhibition of FLT3 pathways in patient blasts. In the patients 11 and 12 blasts with FLT3-ITD, G-749 showed more potent inhibition of p-FLT3 and p-ERK1/2 than AC220 and PKC412. In the patients 13 and 14 blasts with FLT3-D835Y, G-749 also showed more potent inhibition against p-FLT3 than AC220. Both G-749 and PKC412 effectively inhibited p-ERK1/2 in patients 13 and 14 blasts. Remarkably, in cases where they did not inhibit the p-FLT3 (patient 12), AC220 and PKC412 potently inhibited p-STAT5 (Figure 6D). These data suggest that G-749 shows inhibition of p-FLT3 and downstream effectors in patient blasts, regardless of FLT3 mutation status, eventually resulting in efficient antileukemic activity against blasts of AML patients.
DISCUSSION

We report the development of G-749 as a novel FLT3 inhibitor with potent activity against the FLT3 TKD mutants and its sustained inhibition against FLT3 disease pathways was responsible for potent antileukemic activity via apoptosis in AML cells and patient blasts representing different FLT3 mutation status and even in drug resistant environments such as high FL milieu, stroma protecting milieu and relapsed patient plasma. Since it has been proposed that complete and enduring inhibition of FLT3 is critical for achieving clinical efficacy and may contribute to persistence of leukemic progenitors after treatment of FLT3 inhibitors8,9, the prolonged and potent inhibitory activity of G-749 against FLT3 mutants and aberrant downstream pathways could be very effective in treating relapsed and refractory AML patients.

Kinase profiling revealed the potent inhibition of Mer and Aurora B by G-749. Mer kinase is known to be crucial in maintaining cell function but its aberrant levels of TAM receptors and their ligands have been reported in numerous cancers including acute lymphoblastic leukemia (ALL)31-33. Another emerging target for AML treatment is Aurora B that plays an essential role in regulating chromosome segregation and cytokinesis. The inhibition of aurora B, more than aurora A, showed an antiproliferative and pro-apoptotic effect on in vitro and in vivo ALL and AML models34,35. Therefore, the inhibition of aurora B and Mer in addition to inhibition of activated FLT3 disease pathways may contribute to a significant anti-leukemic effect in AML therapy. Other kinases such as FLT1, FMS, and AXL are also known to play a role in the proliferation of hematological malignant tumors36,37 and further studies with them and other possible targets if any remain to be addressed.

FLT3 TKD mutants especially at N676, F691, and D835 were identified to confer resistance to PKC412 and AC22016,19. In this study, we demonstrated potent inhibitory activity of G-749 against FLT3-ITD/N676D and -ITD/F691L and -D835Y mutants. Considering model AML cell lines may have limitation in predicting clinical response of FLT3 inhibitors, we used patient bone marrow blasts.
G-749 showed a dose-dependent cytotoxic response in all leukemic blasts harboring FLT3-WT, -ITD, -D835Y, and -ITD/D835Y. In patient blasts, this cytotoxic activity was well correlated with inhibition of G-749 against p-FLT3 regardless of FLT3 mutant status including those with no or little response to AC220 and PKC412. Therefore, G-749 is expected to potently inhibit FLT3 mutants conferring resistance to other TKIs in clinical study.

The PIA was proposed to be a useful surrogate assay for the determination of FLT3 inhibition in patients receiving oral FLT3 inhibitor because plasma protein binding, which varies between patients, can influence the free drug level necessary for pharmacologic activity. In this study, we demonstrated that G-749 showed significant inhibition of FLT3 and downstream pathways even in relapsed AML patient plasma—this caused significant loss of potency of AC220. We demonstrated that the high FL levels and freely available drug in plasma were not mainly responsible for the differential response of G-749 and AC220 in patient plasma. Rather, this differential could render the hypothesis that the plasma status of patients after the Cytarabine treatment could significantly influence the efficacy of some TKIs targeting FLT3 leading to drug resistance—this remains to be addressed.

A number of studies have been reported that TKIs can rapidly eliminate circulating blasts in peripheral blood. However, this efficacy was restricted to leukemic blasts at the bone marrow probably due to the ability of the bone marrow stroma cells to protect hematological malignancy. In co-culture milieu where bone marrow stroma HS-5 protected patient blasts or MV4-11 cells from dying, G-749 displayed anti-leukemic activity. In the CM from HS-5 where various hematopoietic growth factors significantly altered FLT3 downstream pathways it also showed potent inhibition of ERK1/2 and AKT activations via bypass signals which AC220 did not inhibit. These suggest that the inhibition of FLT3-ERK1/2 axis is indispensible to overcome drug resistance. Overriding the survival bypass signals of leukemic cells provided by the stromal cells, G-749 is expected to yield strong antileukemic activity even in the complex bone marrow microenvironment.
Conclusively, a novel FLT3 inhibitor, G-749 shows potent antileukemic activity in various drug resistant conditions and could be the next generation drug candidate for the treatment of relapsed and refractory AML.
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AUTHORSHIP

Contribution: H.K. Lee designed, conducted experiments and analyzed data; H.W. K. designed, synthesized FLT3 inhibitors for optimization to yield G-749; J.L. conducted kinase assays; I.Y.L. performed cell-based assay; J.L., synthesized chemicals for optimization; D.S.J., S.Y.L., S.H.P. and H.H. conducted animal and pharmacokinetic studies; J.C. and J.K. provided input into compound synthesis; S.W.K., J.K.K. and J.C. provided input into experiments analysis and revised the manuscript; J.S.K. directed chemical synthesis of FLT3 inhibitor project; H. Song directed FLT3 biology project and wrote the manuscript.

Conflict-of-interest disclosure: All authors except for J. C. are current or former employee of Genosco and Oscotec Inc. All authors declare no conflict of interest.

Correspondence: Ho-Juhn Song, Genosco, 767C Concord Ave, Cambridge, MA 02138, USA; e-mail: hsong@genosco.com
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Table 1. Potency comparison of FLT3 inhibitors in biochemical and cellular assays.

Biochemical kinase assay for wild type FLT3 and FLT3-D835Y obtained from Carna Bioscience Inc. was performed in TR-FRET in series concentration of G-749, AC220 and PKC412 and their IC$_{50}$ value was calculated. For the determination of IC$_{50}$ value in human leukemia cell lines, the MV4-11 and Molm-14 cell were treated with test inhibitors and cell viability was measured. The BaF3 model cell expressing double FLT3 mutants, FLT3-ITD/N676D, -ITD/D835Y, -D835Y/N676D and single mutants, FLT3-ITD and FLT3-D835Y were treated with test inhibitors and cell viability was measured.

<table>
<thead>
<tr>
<th>Compound</th>
<th>FLT3 activity (IC$_{50}$, nM)</th>
<th>Human leukemia cell line (IC$_{50}$, nM)</th>
<th>BaF3 cells with FLT3 mutants (IC$_{50}$, nM)</th>
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<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>D835Y</td>
<td>MV4-11</td>
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<tr>
<td>G-749</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>3.5 ± 0.9</td>
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<tr>
<td>AC220</td>
<td>8.8 ± 1.4</td>
<td>28.2 ± 2.1</td>
<td>1.1 ± 0.4</td>
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<tr>
<td>PKC412</td>
<td>15.4 ± 3.5</td>
<td>24.2 ± 3.1</td>
<td>18.5 ± 2.8</td>
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</table>
FIGURE LEGENDS

Figure 1. A novel small molecule FLT3 inhibitor. (A) Chemical structure of G-749. (B) Five human leukemia cell lines were incubated with increasing concentrations of G-749 for 72 hrs. Cell viability was determined using ATPLite assay. G-749 inhibited the proliferation of MV4-11 and Molm-14 with IC₅₀ values of 3.5 and 7.5 nM, respectively. The IC₅₀ values were calculated using nonlinear regression. (C) Molm-14 cells were incubated with the indicated concentrations of G-749 for 2 hrs. The phosphorylation levels of FLT3 (Tyr 591), STAT5 (Tyr 694), ERK1/2 (Tyr 204), AKT (Ser 473) and FoxO3a (Thr 32) were detected by Western blot. Each total protein was used as a loading control. (D) MV4-11 cells were incubated with 100 nM inhibitors for 2 hrs in 10% serum and then washed with fresh medium. The autophosphorylation levels of FLT3 were determined for 24 hrs by phospho-FLT3 ELISA. (E) After washout as in (D), the phosphorylation levels of STAT5 and ERK1/2 were monitored for 8 hrs with treatment of G-749 or AC220. (F) After 18 hrs, caspase-3/7 activities were measured in MV4-11 and Molm-14 cells treated with the indicated concentration of G-749. (G) MV4-11 cells were treated with the indicated concentrations of G-749 for 36 hrs. Cells were stained with propidium iodide/annexin V and then analyzed by flow cytometry. The percentages of early and late apoptotic cells were indicated in the right lower and right upper quadrant, respectively. (H) MV4-11 cells were treated with cytarabine, G-749 or in combination of cytarabine and G-749 (ratio 100:1), and cell viability was measured. Error bars show S.D.

Figure 2. Potent inhibition of FLT3 in various mutant cells. (A) The BaF3 cells expressing the indicated FLT3 mutation were incubated for 2 hrs with the indicated concentrations of G-749 and (B, C) for direct comparison with three inhibitors, the BaF3 cells expressing FLT3-ITD/F691L (B) or -D835Y (C) were incubated with the indicated concentrations of G-749, AC220, and PKC412 for 2 hrs. The autophosphorylation level of FLT3 was visualized and compared by Western blotting analysis.
(D) Molm-14 cells were treated with FLT3 inhibitors in the presence of 5 ng/mL of FL for 2 hrs. The autophosphorylation level of FLT3 was then analyzed. (E) For direct comparison, the phosphorylation levels of FLT3, STAT5, ERK1/2 and AKT were analyzed by immunoblotting as in (D). Total protein of each protein was used as a loading control, otherwise specified.

Figure 3. Potent inhibition of G-749 in AML patient plasma milieu. (A) Molm-14 cells were treated with increasing concentrations of indicated inhibitors in normal culture medium (top), normal human plasma from healthy donor (middle) or normal plasma supplemented with 5 ng/mL of FL (bottom) for 2 hrs. The autophosphorylation level of FLT3 was then evaluated in Western blot. (B) The bone marrow (BM) plasmas were obtained from AML patients who achieved complete remission (CR, n=7) or those with relapse (n=3) after the induction therapy of cytarabine. Molm-14 cells were treated with the indicated concentrations of G-749 or AC220 in relapsed or CR patient plasma milieu for 2 hrs. The p-FLT3 was then analyzed by densitometry and plotted as percentage over DMSO control to display distribution of data. ANOVA followed by Newman-Keuls multiple comparison test was performed to examine the inhibition level of p-FLT3. P-value of <0.05 was calculated between G-749 and AC220 in each concentration for pair-wise comparison. Noticeably, even 1000 nM AC220 treated groups showed greater deviation for inhibition levels of p-FLT3 from 10% to 100% residual p-FLT3 in both relapsed and CR plasma. G-749 showed less deviation for this than AC220 in all tested concentration. (C) The phosphorylation levels of FLT3, STAT5, ERK1/2 and AKT were further evaluated in patient plasma milieu from patient achieving remission (Patient 1, the left side blot) and the relapsed patient (Patient 10, the right side). Noticeably G-749 potently inhibited FLT3 and downstream pathways in both remission and relapsed plasma whereas AC220 significantly lost its potency against them in relapsed plasma.
Figure 4. Stromal protective effect on cell death by FLT3 inhibitors. (A) MV4-11 cells were incubated for 72 hrs with the indicated concentrations of G-749, AC220, and PKC412 in conditioned medium (CM) supplemented with 35% HS-5 derived medium or normal culture medium (NM). Cell viabilities were then determined by test inhibitors and plotted for comparison between NM and CM. (B) AML blasts (patient 12, Supplementary Table 4) were cultured with HS-5 monolayer for 48 hrs with each test inhibitor at 250 and 1000 nM. All cells were stained with Annexin V (x-axis) and CD45 (y-axis) and then gated for CD45 positivity. The percentages of live and apoptotic AML blasts were indicated in upper left and upper right, respectively. (C) Molm-14 cells were incubated for 6 hrs in CM with the indicated concentrations of G-749 or AC220. The phosphorylation levels of FLT3, STAT5, AKT, and ERK1/2 were detected by immunoblotting. Noticeable differential response is that G-749 potently inhibited p-ERK1/2 but AC220 did not.

Figure 5. In vivo anti-tumor activity of G-749 in xenograft and engrafted mouse model. (A, B) Pharmacodynamic analysis of G-749 in a subcutaneous MV4-11 xenograft model. (A) MV4-11 tumor-bearing mice received a single oral dose of G-749 HCl salt (10 mg/kg). Mice were sacrificed at the indicated time point. From homogenized tumor tissues, the levels of p-FLT3 were measured by phosph-FLT3 ELISA in comparison with vehicle-treated control group (Vehicle). The data from 3 mice at each point are presented (± S.D). (B) The p-STAT5 and p-ERK1/2 in the same tumor tissue as in (A) was determined. (C) When the tumor size reached approximately 450 – 600 mm³ in volume, mice (n=9) were orally and qd administered with vehicle or G-749 HCl salt (3, 10 and 30 mg/kg/day) for 28 days. The mice group treated with 30 mg/kg/day of G-749 was subsequently monitored for additional 28 days to examine tumor re-growth. ANOVA with Dunnett’s post-test was performed, *** P<0.0001 at day 28. Significant inhibition of tumor growth and tumor regression was observed from 4 days onward (P <0.05) (D) in vivo anti-tumor activity in an engrafted tumor model. The disseminated NOD/SCID mice were intravenously inoculated with Molm-14 cells. From 7 days after inoculation,
mice were orally and qd given G-749 of 10 or 20 mg/kg/day (n=7) for 28 days. The log-rank test was made to compare survival curves between vehicle treated group and 10 mg/kg/day ($\chi^2 = 13.7$, df=1, P value=0.0002) or 20 mg/kg/day group ($\chi^2 = 13.28$, df=1, P value=0.0003). The gray bars are G-749 dosing period.

Figure 6. Potent inhibition of G-749 against patient blasts harboring FLT3-ITD or -D835Y. (A) AML patient blasts expressing FLT3-WT (patient no. 3), -ITD (patient no. 11 and 12), -D835Y (patient no. 13 and 14) or -ITD/D835Y (patient no. 16) were incubated for 72 hrs with the indicated concentrations of G-749, AC220 or PKC412, and their viability was then determined. For each FLT3 inhibitor, the percentage over DMSO control was presented as a mean value, with error bars representing ± S.D. (B) Inhibition of FLT3 signal pathway. The blasts harboring FLT3-ITD (patient no. 11 and 12) or FLT3-D835Y (patient no. 13 and 14) was incubated with 100 nM of FLT3 inhibitors for 2 hrs (in case of patient no. 12, with 250 nM), and then the phosphorylation levels of FLT3, STAT5, AKT and ERK1/2 were analyzed. Each total protein was used as a loading control.
Figure 1

A

B

C

D

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Figure 2

A

G-749 (nM) 0 1 10 100 1000

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**Culture**

**Plasma**

**Plasma + FL**

B

BM plasma from patients achieving remission

BM plasma from relapsed patients

% p-FLT3 in plasma

G-749 (nM) | AC220 (nM) | G-749 (nM) | AC220 (nM)
Figure 4

A

G-749

AC220

PKC412

Viability (% Control)

Log [G-749] (nM)

Log [AC220] (nM)

Log [PKC412] (nM)

NM

HS-5 CM

B

DMSO

G-749

AC220

PKC412

Annexin V

CD45

250 nM

1000 nM

83.7% 16.2%

76.6% 23.0%

81.5% 18.2%

83.6% 16.3%

60.3% 39.3%

83.2% 16.7%

74.8% 25.2%
Figure 5

A

B

C

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D

![Graph showing percent survival over time for different treatments.](image-url)
Figure 6

A

B

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G-749, a novel FLT3 kinase inhibitor can overcome drug resistance for the treatment of Acute Myeloid Leukemia

Hee Kyu Lee, Hong Woo Kim, In Yong Lee, Jungmi Lee, Jaekyoo Lee, Dong Sik Jung, Sang Yeop Lee, Sung Ho Park, Haejun Hwang, Jang-Sik Choi, Jung-Ho Kim, Se Won Kim, Jung Keun Kim, Jan Cools, Jong Sung Koh and Ho-Juhn Song