Discovery and Characterization of Novel Mutant FLT3 Kinase Inhibitors

Ellen Weisberg1, Hwan Geun Cho2, Rosemary Barrett1, Wenjun Zhou3, Jianming Zhang3, Arghya Ray1, Erik A. Nelson1, Jingrui Jiang1, Daisy Moreno3, Richard Stone1,6,7, Ilene Galinsky1, Edward Fox4, Sophia Adamia1, Andrew L. Kung2, Nathanael S. Gray5, and James D. Griffin1

Abstract

For a subpopulation of acute myeloid leukemia (AML) patients, the constitutively activated tyrosine kinase, mutant FLT3, has emerged as a promising target for therapy. The development of drug resistance, however, is a growing concern for mutant FLT3 inhibitors, such as PKC412. Potential therapeutic benefit can arise from the combination of two structurally diverse inhibitors that target—but bind differently to—the same protein or from two inhibitors with completely different mechanisms of action. Thus, there is a need for identification and development of novel FLT3 inhibitors that have the ability to positively combine with PKC412 or standard chemotherapeutic agents used to treat AML as a way to suppress the development of drug resistance and consequently prolong disease remission. Here, we report the effects of the novel type II ATP-competitive inhibitors, HG-7-85-01 and HG-7-86-01, which potently and selectively target mutant FLT3 protein kinase activity and inhibit the proliferation of cells harboring FLT3-ITD or FLT3 kinase domain point mutants via induction of apoptosis and cell cycle inhibition. Antileukemic activity of HG-7-85-01 was shown in vivo to be comparable with that observed with PKC412 in a bioluminescence assay using NCr nude mice harboring Ba/F3-FLT3-ITD-luc+ cells. HG-7-85-01 was also observed to override PKC412 resistance. Finally, HG-7-85-01 and HG-7-86-01 synergized with PKC412 and standard chemotherapeutic agents against mutant PKC412-sensitive and some PKC412-resistant, FLT3-positive cells. Thus, we present a structurally novel class of FLT3 inhibitors that warrants consideration for clinical testing against drug-resistant disease in AML patients. Mol Cancer Ther; 9(9); 2468–77. ©2010 AACR.

Introduction

Acute myeloid leukemia (AML), which occurs in approximately 10,000 Americans per year, is characterized by aberrant proliferation of myeloid progenitor cells and a partial block in cellular differentiation (1). Approximately 30% of AML patients, and a portion of acute lymphoblastic leukemia patients, harbor a mutant form of the class III receptor tyrosine kinase, FLT3 (fms-like tyrosine kinase-3; STK-1, human stem cell tyrosine kinase-1; or FLK-2, fetal liver kinase-2; ref. 2). Constitutively activated FLT3 occurs most often as internal tandem duplications (ITD) within the juxtamembrane domain (3) and is observed in approximately 20% to 25% of AML patients but in less than 5% of patients with myelodysplastic syndrome (3–8). The transplantation in mice of murine bone marrow cells infected with a retrovirus expressing an FLT3-ITD mutant leads to the development of a rapidly lethal myeloproliferative disease (8).

For a subpopulation of acute myeloid leukemia (AML) patients, the constitutively activated tyrosine kinase, mutant FLT3, has emerged as a promising target for therapy. The development of drug resistance, however, is a growing concern for mutant FLT3 inhibitors, such as PKC412. Potential therapeutic benefit can arise from the combination of two structurally diverse inhibitors that target—but bind differently to—the same protein or from two inhibitors with completely different mechanisms of action. Thus, there is a need for identification and development of novel FLT3 inhibitors that have the ability to positively combine with PKC412 or standard chemotherapeutic agents used to treat AML as a way to suppress the development of drug resistance and consequently prolong disease remission. Here, we report the effects of the novel type II ATP-competitive inhibitors, HG-7-85-01 and HG-7-86-01, which potently and selectively target mutant FLT3 protein kinase activity and inhibit the proliferation of cells harboring FLT3-ITD or FLT3 kinase domain point mutants via induction of apoptosis and cell cycle inhibition. Antileukemic activity of HG-7-85-01 was shown in vivo to be comparable with that observed with PKC412 in a bioluminescence assay using NCr nude mice harboring Ba/F3-FLT3-ITD-luc+ cells. HG-7-85-01 was also observed to override PKC412 resistance. Finally, HG-7-85-01 and HG-7-86-01 synergized with PKC412 and standard chemotherapeutic agents against mutant PKC412-sensitive and some PKC412-resistant, FLT3-positive cells. Thus, we present a structurally novel class of FLT3 inhibitors that warrants consideration for clinical testing against drug-resistant disease in AML patients.

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were observed in 100% of patients harboring mutant FLT3 (13). PKC412 is currently in late-stage, phase III clinical trials. In addition, there are two Food and Drug Administration–approved “multitargeted” kinase inhibitor drugs, sorafenib and sunitinib, which are potent FLT3 inhibitors. Ambit’s AC220 (14) exhibited low nanomolar potency in biochemical and cellular assays, excellent kinase selectivity, and good activity in tumor xenograft model and a mouse bone marrow engraftment model. Millenium Pharmaceuticals’ MLN518 is an orally active inhibitor of FLT3, platelet-derived growth factor receptor α/β (PDGFRα/β), and c-KIT and has shown limited activity as a single agent in phase I/II clinical trials in patients with AML and myelodysplastic syndrome (15).

There are several other reported FLT3 inhibitors, including CGF52421 (metabolite of PKC412), CEP 701, and ABT 869 (16).

Whereas small-molecule inhibitors such as PKC412 are showing promise in the clinic, up to now none has achieved sustained clinical responses as a single agent in AML patients. In addition, a growing problem for the treatment of acute leukemia is the development of resistance due to acquired point mutations in the molecular targets (17, 18). Indeed, the detection of drug-resistant leukemic blast cells in AML patients undergoing PKC412 therapy has sparked the screening and characterization of novel, structurally diverse inhibitors of FLT3 that, if used in combination with antileukemic agents, are predicted to prevent the development of drug resistance.

We report here initial characterization of HG-7-85-01 and HG-7-86-01 as potent and selective inhibitors of mutant FLT3 protein kinase activity. These compounds selectively inhibit FLT3 kinase activity and the proliferation, viability, and cell cycle progression of leukemic cells harboring mutant FLT3, with no apparent effect on cells harboring wild-type FLT3. HG-7-85-01 significantly inhibits mutant FLT3-positive leukemia growth in vivo to a degree comparable with that observed with PKC412. We also show that HG-7-85-01 and HG-7-86-01 effectively combine with PKC412, as well as the standard chemotherapeutic agents 1-/beta-d-arabinofuranosylcytosine (ara-C) and doxorubicin, to kill mutant FLT3-expressing cells to a significantly higher degree than any one agent alone. These results support the notion that FLT3 is an attractive therapeutic target for AML, and point to the emergence of a novel class of inhibitors that could potentially be used in combination with other antileukemic agents to treat mutant FLT3-positive AML.

Materials and Methods

Cell lines and cell culture

The interleukin-3 (IL-3)–dependent murine hematopoietic cell line Ba/F3 was transduced with either FLT3-ITD– or FLT3-D835Y–containing MSCV retroviruses harboring a neomycin selectable marker, and selected for resistance to neomycin (8). FLT3-ITD– and D835Y-expressing Ba/F3 cells were a gift from Dr. Gary Gilliland (Brigham and Women’s Hospital and Dana-Farber Cancer Institute, Boston, MA). Cell lines were obtained proximal to the time of publication of two manuscripts from Dr. James D. Griffin’s and Dr. Gary Gilliland’s laboratories, respectively, in which these constructs were used (8, 12). FLT3 mutations expressed in these lines were confirmed by sequencing, and the entire cDNA was subcloned from pGEM to MSCV-EB neo or MSCV2.2 GFP (kindly provided by W. Pear, University of Pennsylvania, Philadelphia, PA) into the Hpal site in each case. PKC412 responsiveness of the FLT3-ITD– and D835Y-expressing cells was observed to be pharmacologically similar in the current study to results obtained previously with these cell lines (12).

Y572C is a point mutation in the juxtamembrane domain of FLT3 (19). We developed Y572C-expressing Ba/F3 cells in 2004 via transfection using a pCneo-expressing vector, followed by selection for neomycin resistance. The Y572 mutation was confirmed by sequencing using both plasmid and genomic DNA, and the mutation has been characterized as an activating mutation (8).

PKC412-resistant Ba/F3 cell lines expressing FLT3 harboring mutations in the ATP-binding pocket were previously developed (20). These cell lines were developed as a result of identification of FLT3 inhibitor–resistant mutations through vector and library construction and verification of mutant FLT3 sequences (20). The differential PKC412 resistance of these drug-resistant cell lines was investigated and confirmed several times by our lab (21, 22). Cell lines were additionally confirmed pharmacologically to be PKC412 resistant in the current study, with results similar to those observed previously (G697R was observed to be the most highly PKC412 resistant; N676D was observed to be moderately PKC412 resistant in comparison).

We developed the Ba/F3-N841I cell line by transfection using a pCneo-expressing vector, followed by selection for resistance to neomycin (10). We confirmed the sequence of N841I via DNA sequencing. At the time of preparation of ref. 22, the PKC412 responsiveness of Ba/F3 cells expressing the N841I mutant was also confirmed and observed to be similar to what was reported in ref. 10.

The human AML-derived, FLT3-ITD–expressing cell line MOLM13 (German Resource Centre for Biological Material) was engineered to express luciferase fused to neomycin phosphotransferase (pMMP-LucNeo) by transduction with a VSVG-pseudotyped retrovirus as previously described (22, 23). The MOLM13 cell line was confirmed pharmacologically to be PKC412 sensitive in the current study, with drug responsiveness comparable with that previously published (21). The KG-1a cell line, a less well-differentiated variant of the KG-1 cell line, was a gift from Dr. Anthony Letai’s (Harvard Medical School, Boston, MA) laboratory. Both the KG-1 and KG-1a cell lines, originally purchased from the
American Type Culture Collection, showed a similar pharmacologic responsiveness to HG-7-85-01. All cell lines were cultured with 5% CO₂ at 37°C in RPMI 1640 (Mediatech, Inc.) with 10% FCS and supplemented with 1% l-glutamine. Parental Ba/F3 cells were similarly cultured with 15% WEHI-conditioned medium as a source of IL-3. Parental Ba/F3 cells are routinely checked for growth factor dependence by culturing in the absence of WEHI and monitoring viability status. Transfected cell lines were cultured in medium supplemented with 1 mg/mL G418.

Early passages of all lines obtained from the American Type Culture Collection or provided as a gift were expanded and frozen in multiple aliquots at the time of receipt to limit the passage number of the different lines. The cell lines used in this study have not been recently authenticated; however, Dana-Farber Cancer Institute is in the process of doing this now through DNA sequencing and profiling experiments using DNA fingerprinting with small tandem repeat profiling.

**Chemical compounds and biological reagents**

HG-7-85-01 and HG-7-86-01 were synthesized in the laboratory of Dr. Nathanael S. Gray. PKC412 was synthesized by Novartis Pharma AG. Compounds were initially dissolved in DMSO to make 10 mmol/L stock solutions and then were serially diluted to obtain final concentrations of for in vitro experiments. Ara-C and doxorubicin were purchased from Sigma Chemical Co.

**Antibodies and immunoblotting**

Anti-phosphotyrosine (clone 4G10; Upstate Biotechnology) was used at 1:1,000 for immunoblotting. Anti-FLT3/FLK-2 (C-20; Santa Cruz Biotechnology, Inc.) was used at 1:200 for immunoblotting. The monoclonal anti-β-actin antibody (clone AC-15; Sigma-Aldrich) and α-tubulin antibody (clone DM1A; Sigma-Aldrich) were each used at a 1:2000 dilution. The phospho-S6 ribosomal protein (Ser240/244) antibody (Cell Signaling Technology) was used at a dilution of 1:2000. Anti–phospho-signal transducer and activator of transcription 5 (STAT5; Cell Signaling Technology) was used at a dilution of 1:1,000, dilution, and STAT5 (Santa Cruz Biotechnology) was used at 1:10,000 for immunoblotting. Anti–poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology) was used at 1:10,000. The following antibodies were used at a 1:2500 dilution and purchased from Cell Signaling Technology: phospho-mitogen-activated protein kinase (MAPK) and MAPK.

Protein lysate preparation and immunoblotting were carried out as previously described (12).

**Proliferation studies, cell cycle analysis, and apoptosis assay**

Cell counts for proliferation studies were obtained using the trypan blue exclusion assay, as previously described (12). Error bars represent the SE for each data point. Programmed cell death of inhibitor-treated cells was determined using the Annexin-V-Fluos Staining kit (Boehringer Mannheim), as previously described (12). Cell cycle analysis was done as previously described (12).

**Drug combination studies**

For drug combination studies, compounds were added simultaneously at fixed ratios to cells, and cell viability was determined by trypan blue exclusion and expressed as the function of growth-affected drug-treated versus control cells. Synergy was assessed by CalcuSyn software (Biosoft) using the Chou-Talalay method (25). The following formula was used: combination index = [D₁][D₂] + [D₁][D₂][D₃][D₄], where [D₁] and [D₂] are the concentrations required by each drug in combination to achieve the same effect as concentrations [D₃] and [D₄] of each drug alone. Generally, values <1 indicate synergy, whereas values >1 indicate antagonism.

**Mouse studies and in vivo imaging**

Ba/F3-FLT3-ITD cells were transduced with a VSVG-pseudotyped retrovirus composed of the firefly luciferase coding region (from pGL3-basic; Promega) cloned into PMSCV puro (Clonetech). Cells were neomycin selected to produce the Ba/F3-FLT3-ITD (luc+) cell line.

For administration to male NCr nude mice (5–6 weeks of age; Taconic), virus- and Mycoplasma-free cells were washed and resuspended in HBSS (Mediatech) and administered via i.v. tail vein injection (800,000 cells per mouse). Anesthetized mice were imaged 1 to 3 days after i.v. injection to generate a baseline used to establish treatment cohorts with matched tumor burden, and total body luminescence was measured as previously described (24).

**AML patient cells**

Frozen vials of bone marrow from AML patients identified as harboring mutant FLT3 were thawed before processing using Ficoll-Plaque Plus (Amersham Pharmacia Biotech AB) for the isolation of mononuclear cells. Mononuclear cells were isolated from normal bone marrow by density gradient centrifugation through Ficoll-Plaque Plus at 2000 rpm for 30 minutes, followed by two washes in 1× PBS. Mononuclear cells were then tested in liquid culture (Isocove’s modified Dulbecco’s medium, supplemented with 20% FCS) in the presence of different concentrations of HG-7-85-01. All bone marrow samples from AML patients were obtained under approval of the Dana-Farber Cancer Institute Institutional Review Board.

AML patient information is presented as Supplementary Data.

**Results**

**Discovery of HG-7-85-01 and HG-7-86-01 as inhibitors of mutant FLT3**

HG-7-85-01 and HG-7-86-01 were originally designed as a hybrid between the type I inhibitor, dasatinib, and
the type II inhibitor, nilotinib (Fig. 1A). Details about the structure-based design strategy, synthesis of HG-7-85-01, and cocry stallization with Src are described elsewhere (24). Details about the synthesis of HG-7-86-01 are shown in Supplementary Fig. S1.

Kinase selectivity was assessed for HG-7-85-01 and HG-7-86-01 using KINOMEscan (Ambit Biosciences), a high-throughput method for screening kinase inhibitors against a panel of human kinases (26). This technology enables compounds of interest to be assayed for their ability to compete for binding to the ATP site of a panel of 353 kinases, each fused to a proprietary tag. The quantity of each kinase bound to an immobilized, ATP site-directed ligand was measured in the presence and absence of a 10 μmol/L concentration of HG-7-85-01 and HG-7-86-01.

Kinases that displayed >90% displacement relative to the DMSO control were subjected to determination of a dissociation constant (Kd). These studies revealed that, for both compounds, several wild-type and mutant forms of kinases such as Bcr-Abl, PDGFRα/β, FLT3, Ret, Tie-2, Kit, and DDR1 exhibited potent binding (Fig. 1B; Supplementary Table S1; ref. 27). As the results of KINOMEscan suggested that mutant FLT3 may be one of several potential and clinically relevant targets of HG-7-85-01 and HG-7-86-01, this finding required confirmation in cell-based assays.

Effect of HG-7-85-01 and HG-7-86-01 FLT3 kinase activity and FLT3 signaling in mutant FLT3-expressing cells

Both HG-7-85-01 and HG-7-86-01 inhibited mutant FLT3 autophosphorylation in a concentration-dependent manner, with no apparent decrease in mutant FLT3 protein levels (Fig. 1C and D). These results suggest that mutant FLT3 is a direct target of HG-7-85-01 and HG-7-86-01, with HG-7-85-01 showing higher potency than HG-7-86-01. HG-7-85-01 also inhibited phosphorylation of STAT5, an integral component of the Janus-activated kinase/STAT signaling pathway downstream of FLT3, as well as of phospho-S6 ribosomal protein phosphorylation, a component of the mammalian target of rapamycin pathway (Fig. 2A and B; Supplementary Fig. S2C). Similar results were observed with HG-7-86-01, albeit at a
lower potency (Supplementary Fig. S2A, B, and D). HG-7-85-01 also inhibited p44/42 MAPK [extracellular signal-regulated kinase (ERK) 1/2] of the RAS–Raf–MAPK/ERK kinase (MEK)–ERK signaling pathway, another pathway downstream of FLT3 (Fig. 2C). The inhibition by HG-7-85-01 and HG-7-86-01 of multiple components of signaling pathways downstream of mutant FLT3 supports the notion that mutant FLT3 is, indeed, a protein kinase target of these agents and is inhibited as their primary mechanism of action.

Effect of HG-7-85-01 and HG-7-86-01 on proliferation and total cellular tyrosine phosphorylation of cells harboring mutations in the juxtamembrane domain of FLT3

HG-7-85-01 selectively inhibited the growth and viability of FLT3-ITD–expressing MOLM13-luc+ cells and had comparatively little effect toward wild-type FLT3-expressing KG-1 cells or KG-1a cells that were serum starved before FLT3 ligand stimulation (Supplementary Figs. S3A and S4A). HG-7-85-01 potently inhibited the growth of cells expressing mutations in the juxtamembrane domain of FLT3 (IC50 for FLT3-ITD = 2–4 nmol/L; IC50 for Y572C = 1.25–2.5 nmol/L; Table 1), with complete cytoprotection observed when cells were cocultured with drug and WEHI (IL-3), and no effect on the growth and viability of parental Ba/F3 cells at similar concentrations (Supplementary Figs. S3C and D and S4B). The cytoprotective effect of IL-3 suggests that inhibition of mutant FLT3-expressing cells is due to selective inhibition of the mutant FLT3 kinase, and that there is an absence of nonspecific inhibitory effects on growth factor (IL-3)–mediated signaling. HG-7-85-01 also shows activity against AML patient bone marrow samples harboring FLT3 mutations at

Table 1. IC50 values for HG-7-85-01 and HG-7-86-01 against mutant FLT3

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (nmol/L) HG-7-85-01</th>
<th>IC50 (nmol/L) HG-7-86-01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ba/F3-FLT3-ITD</td>
<td>2–4</td>
<td>125–250</td>
</tr>
<tr>
<td>Ba/F3-Y572C</td>
<td>1.25–2.5</td>
<td>12.5–25</td>
</tr>
<tr>
<td>Ba/F3-N841I</td>
<td>12.5–25</td>
<td>200–400</td>
</tr>
<tr>
<td>Ba/F3-D835Y</td>
<td>50–100</td>
<td>500–1000</td>
</tr>
<tr>
<td>Ba/F3-N676D</td>
<td>50</td>
<td>50–100</td>
</tr>
<tr>
<td>Ba/F3-G697R</td>
<td>500</td>
<td>1000–2000</td>
</tr>
</tbody>
</table>

NOTE: IC50 values were calculated based on five concentration linear dose-response curves generated for HG-7-85-01 and HG-7-86-01. Trypan blue exclusion was performed to obtain viable cell counts, and data were plotted as percent of untreated controls.
concentrations ≤ 1 μmol/L (Supplementary Fig. S5A–E), which are concentrations that are both IL-3 resuable and nontoxic toward parental Ba/F3 cells in the murine cell line system. Indeed, one peripheral blood sample tested (AML-35) showed less sensitivity to HG-7-85-01 in the presence of cytokines compared with their absence (Supplementary Fig. S5E), which is similar to the observed IL-3 rescue of mutant FLT3-expressing Ba/F3 cells treated with HG-7-85-01, and supportive of the notion of HG-7-85-01 being selective toward mutant FLT3 as a target. In addition, wild-type FLT3-expressing AML patient samples were generally less sensitive to HG-7-85-01 at concentrations up to 1 μmol/L than AML patient samples expressing mutant FLT3 (Supplementary Fig. S5F).

Similar results were achieved with HG-7-86-01 when tested on cell lines expressing FLT3 juxtamembrane mutations; however, this agent is considerably less potent than HG-7-85-01 (IC50 for FLT3-ITD = 125–250 nmol/L; IC50 for Y572C = 12.5–25 nmol/L; Supplementary Fig. S6; Table 1). Cells were killed by 1 μmol/L HG-7-86-01, with complete protection in the presence of drug when medium was supplemented with growth factor (IL-3), and parental Ba/F3 cells were mostly unaffected in the presence of up to 1 μmol/L HG-7-86-01 (Supplementary Fig. S6).

The ability of HG-7-85-01 and HG-7-86-01 to affect overall cellular tyrosine phosphorylation levels as a measure of selectivity for mutant FLT3 as a target was investigated. HG-7-85-01 and HG-7-86-01 inhibited total cellular tyrosine phosphorylation in FLT3-ITD- and Y572C-expressing cells in a concentration-dependent manner, with no effect on basal levels of tyrosine phosphorylation observed in parental Ba/F3 cells (compared with the higher tyrosine phosphorylation signal in mutant FLT3-expressing cells) and no reduction of levels of FLT3 protein (Supplementary Fig. S7; data not shown). In contrast, levels of FLT3 protein seem to increase in a drug concentration-dependent manner for HG-7-85-01, which is a finding consistent with previous studies carried out with ABL inhibitors such as imatinib and nilotinib (28). These results suggest a possible drug-protein complex formation and stabilization, concurrent with inhibition of kinase activity.

Figure 3. Effect of HG-7-85-01 on viability and cell cycle progression of cells harboring mutations in the juxtamembrane domain of FLT3 in vitro and cell proliferation in vivo. A, induction of apoptosis by HG-7-85-01 of mutant FLT3-expressing cells (following 3 d of treatment). B, effect of HG-7-85-01 on cell cycle progression of mutant FLT3-expressing cells (following a 24-h treatment). C, in vitro effects of HG-7-85-01 on proliferation of cells harboring the FLT3-ITD mutation, shown alongside PKC412 for comparison. D, in vivo effects of HG-7-85-01 on proliferation of cells harboring the FLT3-ITD mutation, shown alongside PKC412 for comparison. This study is representative of two independent studies in which similar results were observed. Approximately 800,000 Ba/F3-FLT3-ITD-luc+ cells were injected into the tail veins of NCr athymic nude mice and treated with either vehicle, PKC412 (100 mg/kg), or HG-7-85-01 (50 mg/kg, 2X/day). Graphed bioluminescence values are shown as percent baseline. Student’s t-test comparison: P < 0.0278, vehicle versus HG-7-85-01 (day 9 after i.v. injection); P < 0.0294, vehicle versus PKC412 (100 mg/kg, day 9 after i.v. injection).
Effect of HG-7-85-01 and HG-7-86-01 on viability and cell cycle progression of cells harboring mutations in the juxtamembrane domain of FLT3 in vitro

We were interested in investigating the underlying mechanism(s) whereby HG-7-85-01 and HG-7-86-01 inhibit the proliferation of mutant FLT3-expressing cells. HG-7-85-01 caused G0-G1 arrest and induced apoptosis of mutant FLT3-expressing cells as evidenced by an increased apoptotic fraction in drug-treated cells, with no effect on viability of parental Ba/F3 cells and no effect on mutant FLT3-positive cell viability in the presence of WEHI (IL-3; Fig. 3A and B; Supplementary Fig. S4B). Similarly, HG-7-86-01 caused G0-G1 arrest and induced apoptosis of FLT3-ITD-expressing cells in the absence of IL-3, with accompanying PARP cleavage, and no effect on viability of parental Ba/F3 cells (Supplementary Figs. S4C and S8). Effects of HG-7-86-01 are selective for mutant FLT3, as evidenced by a lack of toxicity toward normal bone marrow cells in a normal bone marrow colony assay (Supplementary Fig. S8). These results suggest that both induction of apoptosis and inhibition of cell cycle progression contribute to the observed inhibition of cellular proliferation of mutant FLT3-expressing cells by HG-7-85-01 and HG-7-86-01.

Antiproliferative effect of HG-7-85-01 on mutant FLT3-expressing cells in vivo

HG-7-85-01 is ~10-fold more potent than PKC412 against mutant FLT3-positive Ba/F3 cells in vitro (Fig. 3C), although efficacy between the two agents is comparable in vivo (Fig. 3D). In comparison with vehicle-treated mice injected via tail vein with Ba/F3-FLT3-ITD cells, leukemia burden, as assessed by bioluminescence, in drug-treated mice was observed to be significantly suppressed and to a similar degree following 8 days of treatment with HG-7-85-01 (100 mg/kg/d) or PKC412 (100 mg/kg/d), respectively (Fig. 3D). Details about pharmacokinetic characteristics of HG-7-85-01 and HG-7-86-01 are shown in Supplementary Data.

Effect of HG-7-85-01 and HG-7-86-01 on mutant FLT3-expressing cells harboring a point mutation in the activation loop

HG-7-85-01 inhibited the proliferation of cells expressing N841I and D835Y, with the majority of cells killed...
at 1 μmol/L; however, the potency observed was significantly less than that shown against cells expressing FLT3 juxtamembrane domain mutations (IC50 for N841I = 12.5–25 nmol/L; IC50 for D835Y = 50–100 nmol/L; Table 1; Supplementary Fig. S3E and F). HG-7-86-01 showed only weak activity against the N841I and D835Y mutants (IC50 for N841I = 200–400 nmol/L; IC50 for D835Y = 500–1,000 nmol/L; Table 1).

HG-7-85-01 potently inhibited total cellular tyrosine phosphorylation in Ba/F3-N841I and Ba/F3-D835Y cells in a concentration-dependent manner with no reduction of levels of FLT3 protein (Supplementary Fig. S7). HG-7-86-01 inhibited total cellular tyrosine phosphorylation in Ba/F3-N841I cells at 1 μmol/L, with very little effect on Ba/F3-D835Y cellular tyrosine phosphorylation (data not shown).

**Overcoming PKC412 resistance with HG-7-85-01**

In addition to killing PKC412-sensitive mutant FLT3-expressing cells, HG-7-85-01 is able to override some forms of PKC412 resistance (Fig. 4). The PKC412-resistant line Ba/F3-N676D displayed slightly higher sensitivity to HG-7-85-01 than PKC412 (Fig. 4A), as evidenced by a modest shift of the HG-7-85-01 dose-response curve to the left of the PKC412 dose-response curve. More impressively, however, the highly PKC412-resistant line Ba/F3-G697R was sensitive to 1 μmol/L HG-7-85-01, a concentration that does not kill parental Ba/F3 cells and is IL-3 resuable (Fig. 4B and D). In contrast, 1 μmol/L PKC412 kills parental Ba/F3 cells and is not IL-3 resuable, making this concentration of PKC412 nonselectively toxic (Fig. 4D). These results suggest that HG-7-85-01 is able to kill some PKC412-resistant cells with higher potency than PKC412 and/or at concentrations that are physiologically relevant.

**Combination studies: HG-7-85-01 and HG-7-86-01, alone and combined with PKC412 and standard chemotherapeutic agents, ara-C and doxorubicin, against cells harboring FLT3 mutations**

We first investigated the ability of HG-7-85-01 to positively combine with PKC412, ara-C, and doxorubicin against mutant FLT3-positive leukemia. HG-7-85-01 in combination with PKC412 against FLT3-ITD-positive cells was observed to cause a leftward shift in the dose-response curve compared with any one agent alone [CalcuSyn combination indices: 1.03167 (ED50), 0.90664 (ED75), 0.80147 (ED90), or additive to synergistic effects; Table 2]. Combination effects between HG-7-85-01 and doxorubicin similarly suggested synergy, whereas the combination of HG-7-85-01 and the standard chemotherapeutic agent ara-C resulted in values ranging from synergy (ED30) to antagonism (ED20; Table 2).

HG-7-86-01 in combination with PKC412 against Ba/F3-FLT3-ITD and MOLM13-luc+ cells was observed to cause a leftward shift in the dose-response curve compared with HG-7-86-01 alone or PKC412 alone (CalcuSyn: synergy; Table 2). Similarly, HG-7-86-01 in combination with PKC412 led to more killing of Ba/F3-D835Y cells and Ba/F3-Y572C cells than either drug alone (CalcuSyn: synergy; Table 2). The combination between HG-7-86-01 and PKC412 led to more killing of the PKC412-resistant cell line Ba/F3-G697R than either drug alone (CalcuSyn: nearly additive effects to moderate synergism for ED30 to ED50; Table 2). However, antagonism was observed for the combination of HG-7-86-01 and PKC412 against the PKC412-resistant line Ba/F3-N676D. These results suggest that HG-7-86-01 may be able to be effectively combined with FLT3 inhibitors in late-stage clinical development, such as PKC412, in a

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**Table 2.** Combination indices calculated for HG-7-85-01 and HG-7-86-01, respectively, combined with other agents against mutant FLT3

NOTE: Values 0.3-0.7 indicate synergy. Values 0.7-0.85 indicate moderate synergy. Values 0.85-0.9 indicate slight synergy. Values 0.9-1.1 indicate nearly additive effects. Values 1.0-1.2 indicate slight antagonism. Values 1.20-1.45 indicate moderate antagonism. Values 1.45-3.3 indicate antagonism. Those values that indicate moderate synergy to synergy are shaded gray.
nonantagonistic fashion, against mutant FLT3-positive disease and some forms of PKC412-resistant disease.

Lastly, we investigated the combination effects of HG-7-86-01 and the standard chemotherapeutic agents ara-C and doxorubicin against mutant FLT3-expressing cells. The combination of HG-7-86-01 and ara-C led to leftward shifts in the dose-response curves compared with each single agent alone when tested against Ba/F3-FLT3-ITD cells (CalcuSyn: moderate synergism to synergy for ED$_{25}$ to ED$_{90}$). Similar results were observed for the combination of HG-7-86-01 and doxorubicin (CalcuSyn: nearly additive to synergistic results for ED$_{25}$ to ED$_{90}$). These results suggest that HG-7-86-01 could potentially be combined with standard chemotherapy agents, such as ara-C and doxorubicin, in a way that is more efficacious than single agent therapy.

Discussion

Drug resistance in the form of development of point mutations in the target protein is a growing concern in patients treated with tyrosine kinase inhibitors. Combination therapy, using two structurally diverse agents that either bind to different sites on the same protein or interact and inhibit distinct signaling components of the same or different signaling pathways, offers a promising therapeutic strategy designed to suppress the emergence of these point mutations. In the case of chronic myeloid leukemia, combinations of distinct inhibitors of Bcr-Abl, including dasatinib and imatinib, effectively reduced the occurrence of drug-resistant clones (29, 30). Similarly, the combination of imatinib with the dual Src/Abl inhibitors AP23848 and dasatinib, respectively, has shown promise in preclinical in vitro studies involving treatment of nonresistant and imatinib-resistant Bcr-Abl point mutant–expressing cells (31). In the case of PKC412-resistant leukemia, because of the nature of resistance mechanisms, such as emergence of drug-resistant clones (32), it will likely be of significant clinical benefit to simultaneously administer more than one mutant FLT3 inhibitor to patients as a way to suppress the development of these drug-resistant mutants.

FLT3 naturally possesses a phenylalanine residue at the “gatekeeper” position, which has been shown to be responsible for the inability of imatinib to inhibit this kinase (33). The ability of HG-7-85-01 and HG-7-86-01 to potently inhibit FLT3 shows their ability to tolerate large hydrophobic amino acids at the gatekeeper position, consistent with the ability of both compounds to target the T315I mutation of Bcr-Abl. HG-7-85-01 and HG-7-86-01 are distinct amino-thiazolopyridine–based type II ATP-competitive inhibitor. HG-7-85-01 is a low-nanomolar FLT3 inhibitor in biochemical and cellular assays. It is more potent than MLN518, PKC412, CGP5241, and sunitinib and has comparable potency with AC220, CEP 701, and sorafenib as an FLT3 inhibitor. HG-7-85-01 and HG-7-86-01 exhibited considerably greater kinase selectivity than PKC412, CEP 701, CGP52421, sorafenib, and sunitinib. In contrast to HG compounds, the majority of FLT3 inhibitors reported in the literature are type I ATP-competitive inhibitors. Type II ATP-competitive kinase inhibitors recognize the unique DFG-out conformation of kinases and may be advantageous in terms of potency and selectivity as shown in HG compounds and other reported kinase inhibitors (34).

In addition to its antiproliferative effects against gatekeeper mutants (27), HG-7-85-01 selectively induces apoptosis of leukemia cells harboring mutant FLT3 with a significantly higher potency than that of PKC412, with no apparent effect on cells harboring wild-type FLT3. HG-7-85-01 inhibits the proliferation of patient blasts harboring mutant FLT3, is active against PKC412-sensitive and PKC412-resistant isoforms of mutant FLT3, and significantly suppresses FLT3-ITD–induced leukemia burden in mice. HG-7-85-01 also positively combines with PKC412 and standard chemotherapeutic agents, such as ara-C and doxorubicin, to more effectively inhibit the proliferation of mutant FLT3-expressing cells than any one agent alone. The ability of HG-7-85-01 to inhibit mutant FLT3 is a distinctive feature that, in combination with its activity as a type II ATP-competitive inhibitor against a diverse panel of tyrosine kinase gatekeeper mutants (27), underscores its structural uniqueness and potential clinical versatility.

Both compounds were shown to act synergistically with PKC412 to inhibit mutant FLT3-dependent cell proliferation. Thus, either agent could be combined with PKC412 to potentially achieve higher patient responsiveness as well as reduce the likelihood of emergence of some FLT3 point mutations conferring resistance to PKC412. Combinations of mutant FLT3 inhibitors could theoretically be administered on either a sequential, rotating schedule, or administered simultaneously, provided the drugs did not interfere with each other.

HG-7-85-01 and HG-7-86-01 are reasonably selective kinase inhibitors. The selectivity of HG-7-85-01 is intermediate between compounds such as imatinib and nilotinib, which are more selective than HG-7-85-01, and dasatinib, which is less selective (27). Thus, despite the ability of both HG-7-85-01 and HG-7-86-01 to inhibit more than one kinase target (Fig. 1), they are not considered promiscuous kinase inhibitors based on their calculated selectivity score metric (26, 27).

Taken together, these results support the idea that FLT3 is an attractive therapeutic target for AML. Our findings with HG-7-85-01 and HG-7-86-01 also highlight the emergence of a novel class of FLT3 inhibitors that exhibit high selectivity and potency toward mutant FLT3 as an oncogenic target.

Disclosure of Potential Conflicts of Interest

Commercial research grant from Novartis Pharma: J.D. Griffin, R. Stone, A.L. Kung, and N.S. Gray. J.D. Griffin and N.S. Gray have ownership interest in Novartis Pharma and also serve as consultant/advisory board.
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References


# Molecular Cancer Therapeutics

## Discovery and Characterization of Novel Mutant FLT3 Kinase Inhibitors

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